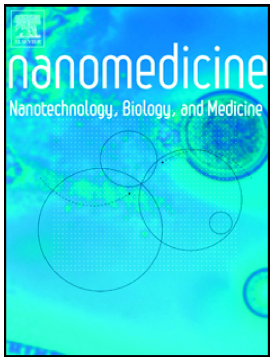
# Setting up and exploitation of a nano/technological platform for the evaluation of HMGA1b protein in peripheral blood of cancer patients

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# Setting up and exploitation of a nano/technological platform for the evaluation of HMGA1b protein in peripheral blood of cancer patients.

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**ABSTRACT**

Even if cancer specific biomarkers are present in peripheral blood of cancer patients, it is very difficult to detect them with conventional technology because of their low concentration. A potential cancer biomarker is the HMGA1b protein, whose overexpression is a feature of several human malignant neoplasias. By taking advantage of the surface plasmon resonance (SPR) phenomenon, we realized a specific nano/technology-based assay for cancer detection. More in details, anti-HMGA1b monoclonal antibodies, whose affinity was previously defined by ELISA, were immobilized onto metallic surfaces to develop a direct SPR-based assay. After having analyzed blood samples from colorectal cancer patients and healthy people for the presence of HMGA1b, we observed a 2-fold increase of the HMGA1b levels in the blood of cancer patients with respect to the healthy control people. We conclude that the set-up technology might allow to detect a tumoral mass through the evaluation of HMGA1b protein blood levels.

**Keywords:** HMGA1b; nano/technology; colorectal cancer; surface plasmon resonance; biosensor.

## BACKGROUND

Colorectal cancer (CRC) represents the second most common cancer in women (9.2% of total cancer cases) and the third in men (10% of total cancer cases), worldwide [1]. About 70% of accounted cases are due to the presence of somatic mutations, whereas 30% of cases rely upon familiar basis [2]. When early detected, surgery is the treatment of choice for CRC. Conversely, when metastatic CRC (mCRC, stage IV disease) is diagnosed, the treatment is generally based on a combination of chemotherapy and biological drugs (monoclonal antibodies) [2]. Even though early detection of CRC could be performed by using different methodologies, more than 50% of cases are diagnosed at a late stage. Therefore, other non-invasive and easy to be performed methodologies are needed for a regular screening of the population that could avoid till 60% of deaths for CRC [3]. At the present, colonoscopy and sigmoidoscopy represent the most frequently used diagnostic tools to assess the occurrence of CRC. They are very sensitive methodologies, but are highly invasive and expensive and, consequently, they cannot be applied for large population screening [4-6]. The faecal immune test (FIT) is also widely used for CRC diagnosis, but it is less sensitive and specific when compared to endoscopic procedures [7].

For visualization of tumors, important advances have recently been made using probes for imaging in the second near-infrared region spectrum, which allows fluorescence images of tumor tissues with superior quality and a low background signal to be obtained [8]. Furthermore, the development of these probes makes possible to reveal them in dual mode even with the use of PET and this represents undoubtedly one of the future scenarios for refining surgical techniques [9, 10]. This detection system is very effective for monitoring fairly pronounced tumor masses and requires patient intervention, even if minimally invasive. On the contrary, the diagnosis based on blood (liquid biopsy) screening makes possible to carry

out the test repeatedly, early, and on very large population sizes. For these reasons, great efforts have been, recently, put into the detection and validation of blood-based protein markers [11-14] that can represent the optimal non-invasive diagnostic strategy also for CRC evaluation [15].

The opportunity of detecting selected biomarkers in the blood is a crucial issue nowadays, since it can help to make important choices in cancer diagnosis, and maybe in therapeutic strategies [16]. A lot of conventional approaches are based on immunologic recognition by ELISA, radio-immunoassay and mass spectrometry [17-19]. However, also these techniques are not applicable for large screening since they are very difficult to implement and routinely perform [16]. Additionally, they are intrinsically affected by a detection limit of picomolar levels, whereas several blood biomarkers are even more diluted in the bloodstream [16, 20]. Therefore, to overcome these troubles, biosensors based on nano/technology are under development and optimization in order to get a quick, rapid and cheap screening of multiple biological liquids, including blood [21-24].

High Mobility Group A (HMGA) are chromatinic proteins mainly characterized by the presence of three AT-hook domains along their *N*-terminal sequence, and a *C*-terminal acidic tail, this latter a characteristic shared with other members of the HMG superfamily (HMGB and HMGN proteins) [25]. HMGA proteins are not/or barely expressed in adult normal tissues, whereas their overexpression is a feature of human malignancies then representing an excellent cancer biomarker [26]. This is one of the characteristics that substantially differentiates them from other proteins of the superfamily (such as HMGB1), which, on the contrary, can also be secreted by immune cells playing an active role in the course of inflammation [25].

Several studies have reported HMGA overexpression in CRC tissues. Indeed, a gradual increase in HMGA expression, going from mild to severe dysplasia, without any detectable expression in non-neoplastic hyperproliferative colonic lesions, has been described

[27-29]. Then, we aimed to the setting-up of a nano-biosensor for the detection of HMGA1b protein in the peripheral blood of cancer patients that could be used for the screening of CRC.

Surface plasmon resonance (SPR)-based biosensors have been widely used as tools for characterizing and quantifying bio-molecular interactions as well as for detection of analytes associated with medical diagnostics. Indeed, they require a simple and fast sample preparation as well as a reduced assay time [30]. As a first step towards the development of a point-of-care health diagnostic tool, we performed an SPR-immunoassay to detect the presence of HMGA1b in the peripheral blood of cancer patients and evaluated its level compared to that detected in healthy people. Our strategy started with a direct assay using monoclonal antibodies, which recognize the HMGA1b epitopes, immobilized on the surface of a $CO_2H_5$ SensiQ chip. Here, we report the assay development and performance of detecting HMGA1b protein in plasma samples.

## METHODS

### Chemicals and reagents

A list of chemicals, reagents and suppliers is reported in Supplementary Methods.

### HMGA1b recombinant protein and antibodies

A detailed description of the expression and purification procedure of HMGA1b recombinant protein is reported in Supplementary Methods.

HMGA1b antibodies used for the construction of the assay and for expression analyses were polyclonal (custom antibody #7393; Neosystem, Strasbourg, France) and monoclonal antibodies (21G6; Areta International, Milan, Italy). The antibody titer was determined by an indirect ELISA assay [31], as reported in detail in Supplementary Methods.

### Western Blot Experiments

BSA and HMGA1b recombinant protein (20 µg for each lane) were separated by SDS-PAGE (15% acrylamide) and then transferred overnight at 4°C, 75 mV, onto a 0.45 µm PVDF Immobilon P membrane (Merck Millipore, Burlington, MA). Membranes were then blocked for 1 h at room temperature in 50 ml of the TBS-T blocking buffer (TBS-Tween 0.05%, containing 5% of milk), washed three times with TBS-T (10 min for each washing) and incubated with monoclonal IgG anti-HMGA1b (1 mg/ml) diluted 1:1,000 in the diluting buffer (TBS-T, containing 1% of milk) for 1 h at 37°C. After three washings with TBS-T (10 min for each washing), membranes were incubated with secondary antibody (goat HRP conjugate, 1:6,000) in the blocking buffer TBS-T for 1 h at room temperature, and washed three times as described above. Finally, proteins were visualized by chemo-luminescence

using the Amersham ECL plus (GE Healthcare, Chicago, IL) and X-ray films manually developed.

Protein extracts were obtained from non-pathological and tumoral tissues deriving from surgical resections and were analyzed for the expression of HMGA1b by Western blot. Protein lysates (50 µg) were separated through SDS-PAGE (15% acrylamide) and then transferred to Immobilon membranes. After blocking in a solution containing TBS-BSA for 1 h, membranes were incubated with HMGA1b and β-Actin antibodies, overnight at 4°C. Subsequently, after three washing with TBS-T buffer, membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, a chemo-luminescent signal was obtained by employing the ECL kit (GE Healthcare) and photographic films were developed and scanned for further analyses.

**Surface plasmon resonance (SPR)**

Real-time measurements of molecular interactions was performed using SPR, which is a quantum-mechanical phenomenon that reflects changes in optical properties at the surface of a sensor chip and eliminates the need to label the interactant molecules. Anti-HMGA1b monoclonal antibodies (HMGA1b mAb) were immobilized on a dextran-layered gold surface and the plasma sample was introduced in a continuous flow over the sensor surface. The SPR detects and measures changes in refractive index due to the binding and dissociation of interacting molecules. The change in refractive index is proportional to the quantity (mass) of analyte interacting with the ligand. The interaction causes a shift in the angle of index at which the SPR phenomenon occurs. The signal is expressed in resonance units (RU), and these shifts, monitored continuously over time, are recorded as a sensorgram. A response of 1,000 RU corresponds to a shift of 0.1° in the response angle, which in turn represents a change in surface protein concentration of about 1 ng/mm$^2$ [32].

The SPR measurements were carried out on SensiQ Discovery instrument (SensiQ Technologies, Inc., Oklahoma City, OK) by using the $CO_2H_5$ sensor chip. All experiments were performed three times at a flow rate of 10 µl/min at room temperature with HBS-E buffer. The data were acquired with SensiQ Discovery software and processing using Qdat Data Analysis software (SensiQ Technologies, Inc.).

***pH Scouting***. To discover the best conditions of antibodies immobilization on the $CO_2H_5$ chip surface, a pH scouting procedure was performed with a SensiQ instrument. Different pH conditions, amount of antibody, time of contact and flow rate were tested. In 10 mM sodium acetate at pH 3.5, 4.0, 4.5, 5.0 and 5.5 the HMGA1b mAb was diluted to a final concentration of 10, 20 and 25 µg/ml. The flow rate tested was 10 and 25 µl/min and a contact time ranging from 5 to 30 min was explored. At the end of each injection, a washing solution (100 mM sodium hydroxide) was injected to remove any unbound molecules from the chip surface. From the sensorgram analysis, the best conditions for the immobilization procedure were chosen.

***Surface preparation***. The carboxy-methylated dextran chip surface was activated by a 1:1 mixture of 0.05 M N-hydroxysuccinamide (NHS) and 0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimidehydrochloride (EDC) pH 7.0 in flow cell 1 and 2. After activation step, HMGA1b mAb were immobilized only on the flow cell 1 (channel 1) of the $CO_2H_5$ chip, while on flow cell 2 (channel 2) a 10 mM sodium acetate pH 4.5 buffer was fluxed. For the immobilization step, the remaining NHS esters were blocked by the injection of a 1.0 M ethanolamine hydrochloride solution at pH 8.5.

***SPR binding measurement (assay format)***. SPR measurements were carried out on the $CO_2H_5$ chip prepared as described above; channel 1 was used as sensing channel because functionalized with HMGA1b mAb and channel 2 was used as reference channel. All sensorgrams automatically acquired by the software, represent the arithmetical difference

between channel 1 and 2. The measures were acquired at room temperature, with a flow rate of 10 µl/min and an injection of 150 µl of the sample on both channels. The regeneration process was performed using 100 mM sodium hydroxide (10 min). The analytical signal of interest was given by the shift in resonance unit (RU) measured as average between two reports point, one fixed at 5 sec before the end of sample injection and second fixed at 5 sec after. A titration curve was obtained by evaluating the binding affinity of the recombinant HMGA1b diluted in 10 mM sodium phosphate buffer (pH 7.3) in a range going from 0 to 7.88 nM. The amount of analyte bound to the receptor was plotted against the analyte concentration, and then the results were fitted by non-linear equation with OriginPro 8 software (Origin Lab, Northampton, MA). All plasma samples collected (healthy people and patients) were analysed by the same assay format utilized to build the calibration curve. All SPR measurements were performed three times, and the obtained results were analysed by Qdat software and OriginPro 8 software.

**Colorectal cancer patients, biological specimens and blood samples processing**

In this study we used samples from 23 patients undergoing surgery for resection of CRC mass and 8 healthy peoples. Patients were hospitalized at University Hospitals of Naples for the surgical removal of the tumor starting from May 2014 (Supplementary Table S1). The day of the surgery a blood sample was obtained from each CRC patient. The same volume of blood was obtained by volunteer healthy people controls (Supplementary Table S2). From the same patients, the day of the surgery small pieces of tumor tissues and corresponding non-pathological tissues from a site distant from the neoplasia were also obtained. These biological fragments were immediately processed and stored a -80°C for further extraction of proteins and nucleic acids. Disruption of tissues and extraction of biological macromolecules was performed as previously reported [33]. Five ml of blood samples were processed for plasma

evaluation. Briefly, blood placed in a green cap vacutainer tube (Becton Dickinson, Franklin Lakes, NJ) was centrifuged for 10 min at room temperature and 2,100 rcf, to remove red cells. The supernatant was recovered, subdivided in small volume aliquots and finally stored at -80°C for further analyses.

**Quantitative Real-Time PCR analysis**

RNA samples obtained from non-pathological and tumoral tissues were used to generate cDNA. Subsequently, 10 ng of cDNA was used for quantitative RT-PCR analysis of HMGA1b expression. Reactions were performed in triplicate on a C1000/CFX96 instrument (Biorad, Hercules, CA). We used TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA) for the evaluation of HMGA1b (Assay ID Hs00852949_g1, Catalog # 4331182) and internal reference β-Actin (Assay ID Hs01060665_g1 ACTB, Catalog # 4331182) gene.

Reactions were carried out with the following thermal protocols, 95°C for 10 min (initial denaturation), 95°C for 30 sec (cycle denaturation) and 60°C for 1 min (cycle denaturation and extension) for 40 cycles, 72°C for 10 min (final extension). Raw data were processed with CFX Manager Software (Biorad) and expression results were shown as relative expression of tumoral sample compared to each corresponding non-pathological sample, according to the $2^{-\Delta\Delta Ct}$ formula.

**Statistical analysis**

Raw data were processed and analyzed through the use of different softwares, specifically indicated where appropriate. Statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA). Comparisons were carried-out by

using the appropriate test and $p<0.05$ was considered statistically significant. Correlation analyses were performed by using Spearman and Pearson r.

SPR data were processed by Origin Pro 8 software; the mean and SD of each samples were calculated. The Shapiro-Wilk test was used to evaluate the normal distribution of acquired data. The mean of control group and patients group was compared by two-side *t*-test applied $p<0.01$ as cut-off of statistical meaningfulness.

**Ethics**

Research was conducted according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from patients involved in the study.

## RESULTS

### Production of HMGA1b recombinant protein

A large amount of HMGA1b recombinant protein (P17096-2) was produced as reported in Supplementary Methods. After induction and purification, HMGA1b recombinant protein underwent quality control procedure as reported below. The HMGA1b protein, purified to the homogeneity (Figure 1A), was evaluated by SDS PAGE (15% acrylamide). Final purified protein yield (2 mg per g of humid pellet) was calculated by Lambert and Beer's law using molar extinction coefficient equal to 67,500 $M^{-1}$ $cm^{-1}$. Western blot analysis confirmed the presence of purified HMGA1b (Figure 1B).

### ELISA test

To evaluate the specificity and the titer of the polyclonal (pAb) and monoclonal (mAb) antibodies, an indirect ELISA test was performed. For this purpose, we coated the micro-plate wells with different concentrations of the HMGA1b recombinant antigen, and tested serially diluted pAb and mAb against produced HMGA1b. For non-coated wells, no signal was registered as a consequence of incubation with different diluted samples of IgG. The obtained results (Figure 1C) displayed the specificity and high quality of the titer of HMGA1b mAb. In fact, it was possible to perform the ELISA test with antibodies dilutions from 1 to 100,000. Additionally, a good response was detected on coated HMGA1b at a concentration of 500 ng/ml.

### Surface plasmon resonance (SPR) experiments

The next step was to build up a direct SPR immuno-assay up to reduce time and cost of analysis of HMGA1b in the plasma of cancer patients. HMGA1b mAb was used to enhance specificity and sensitivity of the performance. Furthermore, with the aim to develop a point-of-care health diagnostic tool, the assay was performed on the portable instrument SensiQ Discovery (SensiQ Technologies, Inc.).

Based on the ELISA test, the HMGA1b mAb were selected as bio-specific ligand to covalently immobilize on the $CO_2H_5$ sensor surface. A pre-concentration test was performed in order to identify the best condition of immobilization. HMGA1b mAb was diluted at three concentrations (10, 20 and 25 µg/ml) in 10 mM sodium acetate buffer at variable pH comprised in the range 3.5-5.5 and fluxed onto sensor surface. Furthermore, two flow rates of injection (10 and 20 µl/min) and a contact time from 5 to 30 min were explored. According to the results obtained with the pre-concentration test, we chose the best immobilization conditions that consists a mAb solution of 25 µg/ml in 10 mM sodium acetate at pH 4.5 with a flow rate of 10 µl/min and a contact time of 30 min. Under these conditions the immobilization procedure was performed in three steps: (a) surface activation with 70 µl of EDC/NHS pH 7.0 mixtures with a flow rate of 10 µl/min; (b) covalent binding of mAb to the surface (300 µl); (c) deactivation of any remaining active esters by ethanolamine (10 min). Results of immobilization are shown in Figure 2A. As we can observe, the amount of HMGA1b mAb immobilized on the chip surface corresponded to 4,335 RU.

**SPR binding measurement (assay format)**

Binding experiments were performed to evaluate the affinity of HMGA1b to the corresponding mAb immobilized on the chip surface. Binding capacity was monitored as function of time.

The sensorgram depicted in Figure 2B shows the variation of the RU, as a function of the time, in the absence and presence of a wide range of concentrations of recombinant HMGA1b (0 to 7.88 nM). From the analysis of data, it is evident that the RU signal increases with increasing concentrations of HMGA1b as a consequence of binding on the surface. By Qdat software analysis, a Kd of 2±0.2 nM was calculated.

As reported in Figure 2C and Table 1, RU values acquired from the titration experiments and calculated as average between the last 5 sec of association phase and the first 5 sec of dissociation phase were plotted in function of HMGA1b concentration. Through the use of non-linear fitting (Hill's equation), a calibration curve was built, and the last one was used to evaluate the HMGA1b concentration in plasma samples.

The limit of blank (LOB) and detection (LOD) of the assay were calculated according to Armbruster and Pry [34], and it is possible to conclude that the described method displayed a LOB of 31.48 pM corresponding to 36.75 ng/dl and a LOD of 51.20 pM corresponding to 59.78 ng/dl. Once established the assay format and its performance, the last one was used to evaluate the presence of HMGA1b in the plasma of patients and healthy control people.

**SPR plasma sample measurements**

The collected plasma samples were diluted 1:500 in 10 mM sodium phosphate pH 7.3 and tested on sensor chip. A triplicate measurement of each sample (Supplementary Figures S1-S4) was acquired, and the mean and standard deviation were calculated for each sample (Table 2 and Table 3). All the results were plotted in Figure 3A.

Mean RU value corresponding to the control and patients group were normalized by patients group mean value and plotted in Figure 3B where we can observe a statistical significant difference between the two groups (*t*-test: *p=0.0034*).

In Table 2 and Table 3 we also reported the values of circulating HMGA1b obtained from calibration curve (mg/dl). Briefly, from the fitted curve (applying the fitting equation reported in Figure 2C) we extracted the millimolar concentration values, multiplying the last one for the dilution factor (500), for molecular weight of HMGA1b and divide for ten. From this analysis, the amount of circulating HMGA1b in the control group was comprised in a range going from 0.306 to 0.506 mg/dl, with a mean value of 0.406±0.072 mg/dl, while in the patient group it was comprised in a range going from 0.350 to 1.977 mg/dl with a mean value of 0.821±0.464 mg/dl.

**Clinical-pathological characteristics of patients**

The main clinical-pathological characteristics of 23 CRC patients recruited for this investigation are reported in Supplementary Table S1. Additionally, eight healthy people were involved in this study (Supplementary Table S2). The age of these people at the bleeding is indicated in Supplementary Table S1 and S2. For privacy requirements, a consecutive number was assigned to each patient and control. As far as cancer patients characteristics is concerned, we have found information for 11 patients. Diagnosis was of adenocarcinoma for all the patients and the staging essentially reported that these carcinomas are pT3 (pT3, 9 out of 11 patients; pT4, 2 out of 9 patients) and well differentiated (G1, 9 out of 11 patients; G3, 2 out of 11 patients) (Supplementary Table S1). As far as the lymph node involvement is concerned, 2 patients were N1, 2 patients were N2, and the remaining 7 patients were N0 (Supplementary Table S1).

**Evaluation of HMGA1b expression in pathological tissues**

Since our objective was to evaluate the occurrence of cancer based on the presence of HMGA1b in the blood of cancer patients, we evaluated HMGA1 expression in primary CRC mass by qRT-PCR and Western blot analyses.

We were able to analyse by qRT-PCR 17 samples out of 23 ones used for biosensor analysis. We found HMGA1b overexpressed in 13 out of 17 samples analysed (76%), considering a relative expression value ≥2 (Figure 4A). One sample out of 17 analysed (6%) showed a relative expression value ≤0.5 (underexpressed), while 3 out of 17 samples (18%) showed a relative expression value ≥0.5 and ≤2.0 (unchanged). Relative expression values were calculated by considering matched tumoral and non-pathological corresponding tissues, as reported in Methods section. In the same tissue fragments we also evaluated the overexpression of HMGA1b protein by Western blot analysis and found that HMGA1b was overexpressed in tumoral tissues (Figure 4B). We were able to analyse 19 samples of the 23 ones used for the biosensor analysis and, through densitometric analysis, we found that in 7 out of 19 samples analysed (36.9%) HMGA1b protein resulted overexpressed with a relative expression value ≥2, when compared with matched non-pathological colonic mucosa (Figure 4B). By applying the same rules, 10 out of 19 samples (52.6%) resulted unchanged (relative expression value ≥0.5 and ≤2.0) and 2 out of 19 samples analysed (10.5%) were underexpressed (relative expression value ≤0.5). β-Actin protein was evaluated to normalize the amount of protein loaded in each lane and densitometric analysis was performed through the use of Image J software (National Institutes of Health, Bethesda, MD), as reported in Methods section.

Subsequently, we correlated the expression results obtained by qRT-PCR and Western blot analyses. A statistical significant correlation (Supplementary Table S3, Figure 4C) between the two methodologies was observed (Spearman r: r=0.5245, *p=0.0327*).

Finally, we also performed a correlation analysis between qRT-PCR and Western blot results with the values of circulating HMGA1b as measured through the employment of the biosensor (Supplementary Table S3, Figure 5). Unfortunately, in this case, we did not observe any statistical significant correlation (Biosensing assay *vs* Western blot, Pearson r: r=-0.4154, *p=0.0769*; Biosensing assay *vs* qRT-PCR, Pearson r: r=-0.0758, *p=0.7725*).

## DISCUSSION

Cancer is a very common disease, especially in the most developed countries, and despite recent advances in personalized therapy, early prevention and diagnosis are yielding much more encouraging results. In this perspective, there is a great interest of the scientific community regarding the possibility of early detection of cancer using liquid biopsy and minimally invasive studies. In fact, to make a very accurate diagnosis of carcinoma, it is often necessary to perform invasive and painful procedures for the patient.

Nano/technology is a very active field in medicine sciences and multiple biosensors have been developed to monitor the presence and course of cancer. They do not rely only on the presence of canonical biomarkers such as proteins, but they also explore the evaluation of microRNAs in biological fluids as markers of cancer [35]. Furthermore, they explore different physical-chemical properties based on optics, electricity and microfluidics.

In this study, we report the possibility to sense HMGA1b, a putative marker of colorectal cancer in the peripheral blood of patients. To achieve this aim we have built a nano/technology-based biosensor capable of detecting the presence of HMGA1b in the peripheral blood with high specificity and sensitivity. This is possible thanks to the availability of a monoclonal antibody able to recognize human HMGA1b protein with high affinity. Surface plasmon resonance has been successfully exploited to detect with high sensitivity and specificity small amounts of circulating HMGA1b, generally overexpressed in cancer tissues, but not in the corresponding tissues from healthy people. In fact, we have successfully reported that our biosensing assay is able to detect higher levels of HMGA1b protein in the plasma of CRC patients, if compared with control healthy people. We were able to detect a mean 2-fold increase of the HMGA1b protein levels in the plasma obtained from CRC patients, in comparison with plasma samples deriving from healthy people.

Unfortunately, we did not observe a statistical correlation between expression results obtained by the use of biosensor and qRT-PCR/Western blot. This could probably mean that the values of circulating HMGA1b detected in the blood are depending on other clinical-pathological features of the tumors such as size of the tumor, stage and grade, other than the simple overexpression of the HMGA1 alone.

Clearly, other biological fluids can also be analyzed for an early diagnosis perspective. For example, saliva and urine are the most widely used biological fluids besides blood. In the future, we have planned to extend the evaluation of HMGA1b to urine, as in the case of bladder cancer.

Obviously, in order to monitor the presence of cancer by means of liquid biopsies, it is necessary that the examined biomarker is present in it and derives from the tumor. HMGA1b is an excellent example of a biomarker since it is present at low levels in healthy adult tissues, but it is abundantly expressed in embryonic tissues and in the majority of human carcinomas [26]. To cite an instance, several studies have reported that the mRNA corresponding to HMGA proteins is highly present in the blood of breast and ovarian cancer patients [36-38].

Certainly, our study has some limitations as it has been performed on a limited number of patients. Moreover, the evaluations of HMGA1b were not carried out along the course of the disease and during subsequent therapeutic treatments, but our results, although preliminary, are very promising and validate the ability to monitor the levels of HMGA1b protein and to associate them with the early presence of cancer. Therefore, to answer to these questions, our future goal is to assess the presence of HMGA1b during the therapeutic treatment and to monitor the presence of relapse. This would allow not only to monitor the patient's health conditions in real time, but also to reduce the costs necessary for diagnostic tests. One of the future goal is to build an integrated and miniaturized device to make the assay usable in public diagnostic facilities.

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## FIGURE LEGENDS

**Figure 1. Quality evaluation of purified recombinant HMGA1b. Specificity evaluation of HMGA1b mAb *vs* HMGA1b pAb.**

**(A)** 15% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, BSA; lanes 3 and 4 recombinant HMGA1b protein (in lane 4 half amount); lane 5, Arg BP (arginine binding protein as negative control). **(B)** Western blot x-ray obtained from SDS-PAGE described in Figure 1A, HMGA1b mAb was used to detect the presence of HMGA1b in lanes 3 and 4. **(C)** Indirect ELISA test to compare titer and specificity of the rabbit HMGA1b pAb *vs* mouse HMGA1b mAb. Each Ab shows a powerful titer, a good response was obtained at a dilution of 1:100,000, but mAb shows a minor background level (no coating signal was low) and a minor response to BSA protein.

**Figure 2. SPR assay setting up and calibration curve construction.**

**(A)** Sensorgram of amine coupling immobilization procedure. Three steps are shown: (1) activation with EDC/NHS mixture, (2) bound of HMGA1b mAb and (3) ester deactivation. mAb was immobilized at 4,335 RU. **(B)** SPR binding experiments. Sensorgram showing the binding of recombinant HMGA1b protein to HMGA1b mAb covalently immobilized on the sensor chip surface. All measurements were performed in 10 mM sodium phosphate buffer (pH 7.3) at room temperature. **(C)** Calibration curve. Titration of the SPR-based sensing system with HMGA1b in 10 mM sodium phosphate buffer (black squares). RU values are plotted versus HMGA1b concentration; red curve shows a result of non-linear fitting operation. Kd value calculated by Qdat software was 2±0.2 nM.

**Figure 3. Healthy people and colorectal carcinoma patient plasma samples SPR measurements.**

**(A)** Graph reports the mean and standard deviation (SD) RU values of each sample analysed; the mean and SD was obtained from a triplicate measure. Data obtained from patients group (S1-S23) were plotted in blue, while data obtained from healthy people group (CTRL1-CTRL8) were plotted in red. **(B)** In this graph the normalized RU mean values of each group, patients and control, were compared (*t*-test, p=0.0034).

**Figure 4. HMGA1b overexpression in colorectal carcinoma samples.**

**(A)** qRT-PCR analysis of HMGA1b expression in matched tumoral (T) and non-pathological (NT) tissues. β-Actin gene was used as internal reference gene and relative expression was calculated by applying the $2^{-\Delta\Delta Ct}$ formula. Relative expression values were obtained by setting equal to 1 the expression value corresponding to each normal sample. **(B)** Western blot analysis of HMGA1 protein in matched tumoral (T) and non-pathological (NT) tissues. Immuno-blots were performed by employing a polyclonal antibody raised against the human HMGA1 protein. β-Actin was used to normalize the amount of protein loaded onto each channel of the gel (lower panel). Densitometric analysis of HMGA1 overexpression was performed by using the Image J software. Intensities of HMGA1 band were divided for the intensities corresponding to β-Actin. Then, normalized values obtained in tumoral samples were divided for each corresponding normalized values of non-pathological tissue, obtaining the relative expression values (upper panel). **(C)** Correlation analysis between Western blot and qRT-PCR results. Statistical correlation and linear regression were calculated by using the GraphPad Prism software. Spearman r: r=0.5245, *p=0.0327*.

**Figure 5. Correlation analysis between Biosensing assay, Western blot and qRT-PCR and results.**

Statistical correlation and linear regression were calculated by using the GraphPad Prism software. Biosensing assay *vs* Western blot, Pearson r: r=-0.4154, *p=0.0769*; Biosensing assay *vs* qRT-PCR, Pearson r: r=-0.0758, *p=0.7725*.

**Table 1.** Raw data SPR measurements of recombinant HMGA1b.

| [HMGA1b] µg/dl | [HMGA1b] nM | Response (RU) | | | | |
|---|---|---|---|---|---|---|
| | | T1 | T2 | T3 | M | SD |
| **0** | **0** | 15,39 | 3,18 | 10,61 | 9,73 | 6,15 |
| **0,367** | **0,315** | 371,78 | 352,97 | 352,97 | 359,24 | 10,86 |
| **0,735** | **0,630** | 928,74 | 880,39 | 1014,52 | 941,22 | 67,93 |
| **1,471** | **1,26** | 1126,90 | 997,06 | 1224,71 | 1116,22 | 114,20 |
| **2,942** | **2,52** | 1913,90 | 1856,54 | 2130,39 | 1966,94 | 144,43 |
| **5,885** | **5,04** | 2541,53 | 2516,71 | 2611,69 | 2556,64 | 49,26 |
| **9,201** | **7,88** | 2628,29 | 2538,75 | 2677,98 | 2615,01 | 70,56 |

**Table 2.** Raw data SPR experiments and statistical analysis of healthy people plasma samples.

| PLASMA SAMPLE | Response (RU) | | | | | [mg/dl] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | M | SD | T1 | T2 | T3 | M | SD |
| CTRL 1 | 668,91 | 668,08 | 631,81 | 656,27 | 21,18 | 0,312 | 0,311 | 0,295 | 0,306 | 0,010 |
| CTRL 2 | 817,25 | 763,09 | 803,31 | 794,55 | 28,12 | 0,381 | 0,355 | 0,374 | 0,370 | 0,013 |
| CTRL 3 | 1036,41 | 1081,32 | 1006,85 | 1041,53 | 37,50 | 0,495 | 0,520 | 0,478 | 0,498 | 0,021 |
| CTRL 4 | 851,25 | 804,75 | 791,20 | 815,73 | 31,50 | 0,398 | 0,375 | 0,368 | 0,380 | 0,015 |
| CTRL 5 | 1073,98 | 1009,34 | 1087,40 | 1056,91 | 41,74 | 0,516 | 0,480 | 0,523 | 0,506 | 0,023 |
| CTRL 6 | 745,23 | 737,55 | 729,75 | 737,51 | 7,74 | 0,347 | 0,343 | 0,339 | 0,343 | 0,004 |
| CTRL 7 | 942,13 | 955,13 | 964,13 | 953,80 | 11,06 | 0,444 | 0,451 | 0,455 | 0,450 | 0,006 |
| CTRL 8 | 856,72 | 856,16 | 805,80 | 839,56 | 29,24 | 0,400 | 0,400 | 0,376 | 0,392 | 0,014 |
| M | | | | 861,98 | | | | | 0,406 | 0,013 |
| SD M | | | | 143,25 | | | | | 0,072 | |

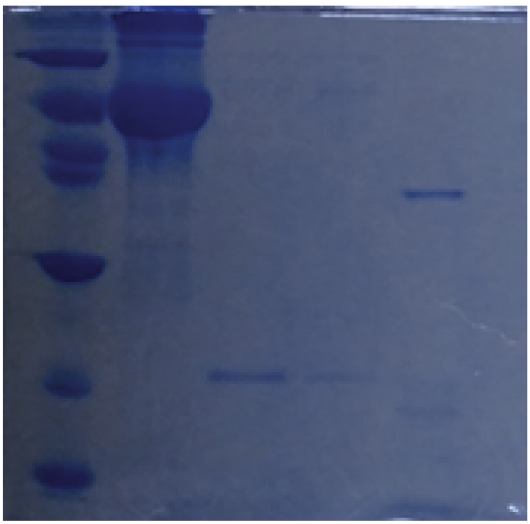
CTRL=control (healthy people); T=test; M=mean; SD=standard deviation; SD M=standard deviation of mean

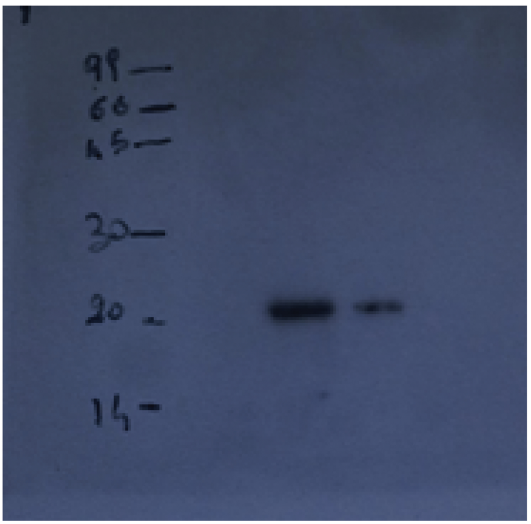
**Table 3.** Raw data SPR experiments and statistical analysis of patient plasma samples.

| PLASMA SAMPLE | Response (RU) | | | | | [mg/dl] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | M | SD | T1 | T2 | T3 | M | SD |
| S1 | 1147,46 | 1113,40 | 1138,68 | 1133,18 | 17,68 | 0,559 | 0,539 | 0,553 | 0,550 | 0,010 |
| S2 | 1184,85 | 1166,10 | 1121,18 | 1157,38 | 32,72 | 0,582 | 0,570 | 0,543 | 0,565 | 0,020 |
| S3 | 1044,03 | 1139,14 | 1078,74 | 1087,30 | 48,13 | 0,499 | 0,554 | 0,518 | 0,524 | 0,028 |
| S4 | 1608,33 | 1914,61 | 1862,75 | 1795,23 | 163,92 | 0,902 | 1,255 | 1,184 | 1,113 | 0,186 |
| S5 | 2131,56 | 2041,54 | 2035,67 | 2069,59 | 53,75 | 1,631 | 1,456 | 1,446 | 1,511 | 0,104 |
| S6 | 1637,06 | 1545,62 | 1468,99 | 1550,56 | 84,14 | 0,929 | 0,845 | 0,781 | 0,852 | 0,074 |
| S7 | 2400,46 | 2253,51 | 2101,83 | 2251,93 | 149,32 | 2,434 | 1,928 | 1,570 | 1,977 | 0,434 |
| S8 | 1056,93 | 1045,61 | 1055,95 | 1052,83 | 6,27 | 0,506 | 0,500 | 0,506 | 0,504 | 0,004 |
| S9 | 1882,29 | 2024,39 | 1922,51 | 1943,06 | 73,25 | 1,210 | 1,426 | 1,266 | 1,301 | 0,112 |
| S10 | 962,98 | 944,72 | 981,27 | 962,99 | 18,28 | 0,455 | 0,445 | 0,465 | 0,455 | 0,010 |
| S11 | 1372,64 | 1395,43 | 1415,87 | 1394,65 | 21,63 | 0,708 | 0,724 | 0,740 | 0,724 | 0,016 |
| S12 | 1776,26 | 1792,64 | 1744,83 | 1771,24 | 24,30 | 1,077 | 1,096 | 1,041 | 1,071 | 0,028 |
| S13 | 739,68 | 717,95 | 796,02 | 751,22 | 40,29 | 0,344 | 0,334 | 0,371 | 0,350 | 0,019 |
| S14 | 1073,07 | 1212,41 | 1238,59 | 1174,69 | 88,97 | 0,515 | 0,599 | 0,616 | 0,577 | 0,054 |
| S15 | 825,02 | 792,76 | 801,16 | 806,31 | 16,74 | 0,385 | 0,369 | 0,373 | 0,376 | 0,008 |
| S16 | 1602,22 | 1550,81 | 1579,11 | 1577,38 | 25,75 | 0,896 | 0,850 | 0,875 | 0,874 | 0,023 |
| S17 | 1179,22 | 1154,85 | 1126,29 | 1153,45 | 26,49 | 0,578 | 0,563 | 0,546 | 0,562 | 0,016 |
| S18 | 1587,44 | 1575,50 | 1646,47 | 1603,14 | 38,00 | 0,883 | 0,872 | 0,939 | 0,898 | 0,036 |
| S19 | 2344,76 | 2168,19 | 2167,09 | 2226,68 | 102,26 | 2,217 | 1,712 | 1,709 | 1,879 | 0,292 |
| S20 | 1213,63 | 1209,67 | 1176,58 | 1199,96 | 20,34 | 0,600 | 0,597 | 0,576 | 0,591 | 0,013 |
| S21 | 935, | 900, | 900, | 912, | 19,8 | 0,44 | 0,42 | 0,42 | 0,42 | 0,01 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 23 | 71 | 91 | 28 | 7 | 0 | 3 | 3 | 9 | 0 |
| **S22** | 1539,20 | 1499,22 | 1456,10 | 1498,17 | 41,56 | 0,840 | 0,806 | 0,771 | 0,806 | 0,034 |
| **S23** | 913,79 | 816,42 | 804,80 | 845,00 | 59,85 | 0,429 | 0,381 | 0,375 | 0,395 | 0,030 |
| **M** | | | | 1387,75 | | -- | | | 0,821 | |
| **SD M** | | | | 455,19 | | | | | 0,464 | |

S=patient; T=test; M=mean; SD=standard deviation; SD M=standard deviation of mean




98
60
45
30
20
14

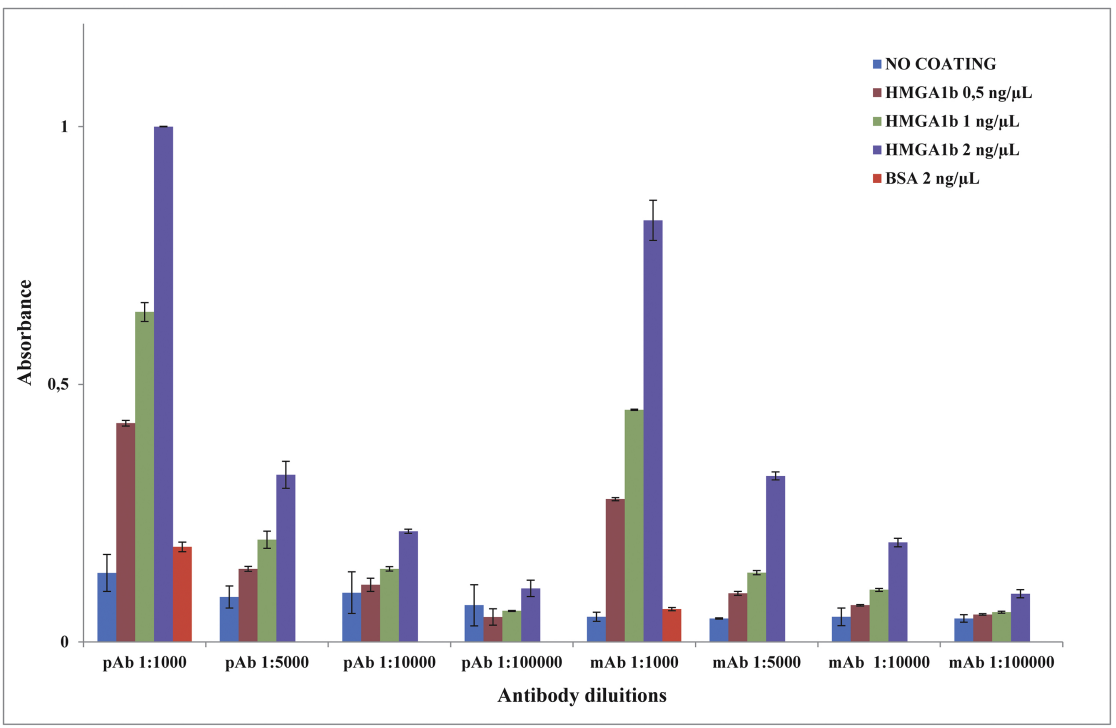
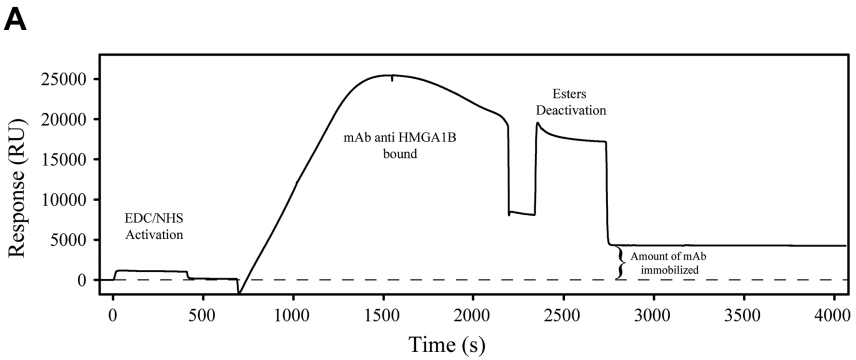
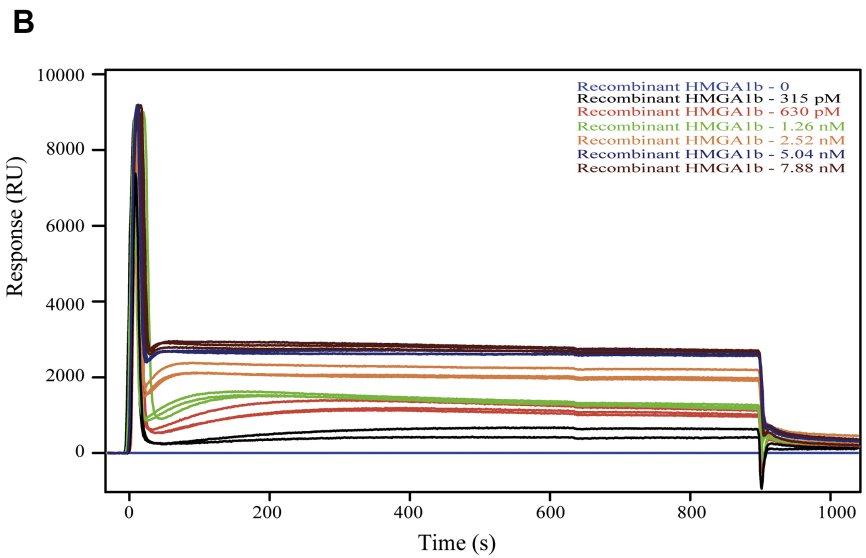

NO COATING
HMGA1b 0,5 ng/µL
HMGA1b 1 ng/µL
HMGA1b 2 ng/µL
BSA 2 ng/µL
Absorbance
1
0,5
0
pAb 1:1000
pAb 1:5000
pAb 1:10000
pAb 1:100000
mAb 1:1000
mAb 1:5000
mAb 1:10000
mAb 1:100000
Antibody diluitions

Figure 1


A
Response (RU)
Time (s)
EDC/NHS
Activation
mAb anti HMGA1B
bound
Esters
Deactivation
Amount of mAb
immobilized


B
Response (RU)
Time (s)
Recombinant HMGA1b - 0
Recombinant HMGA1b - 315 pM
Recombinant HMGA1b - 630 pM
Recombinant HMGA1b - 1.26 nM
Recombinant HMGA1b - 2.52 nM
Recombinant HMGA1b - 5.04 nM
Recombinant HMGA1b - 7.88 nM

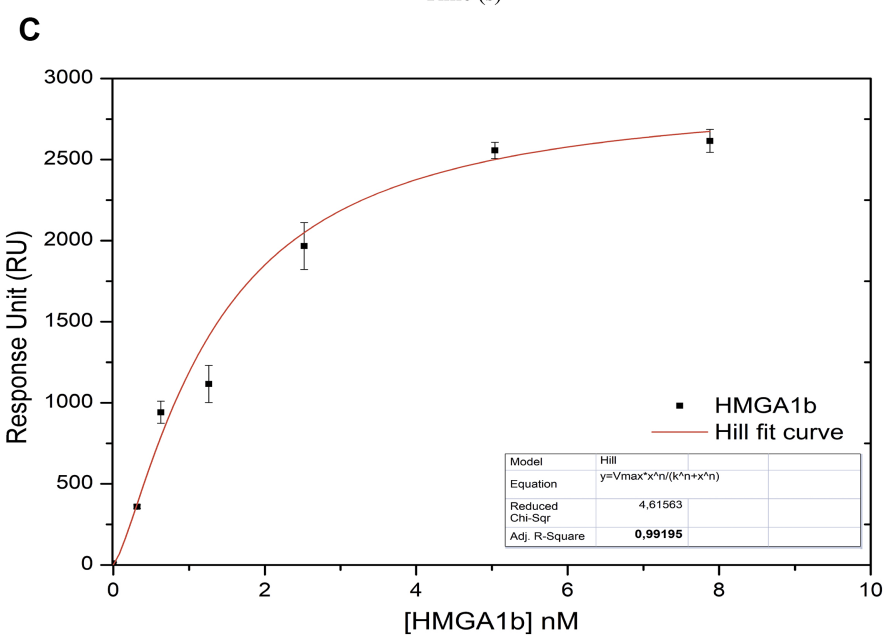
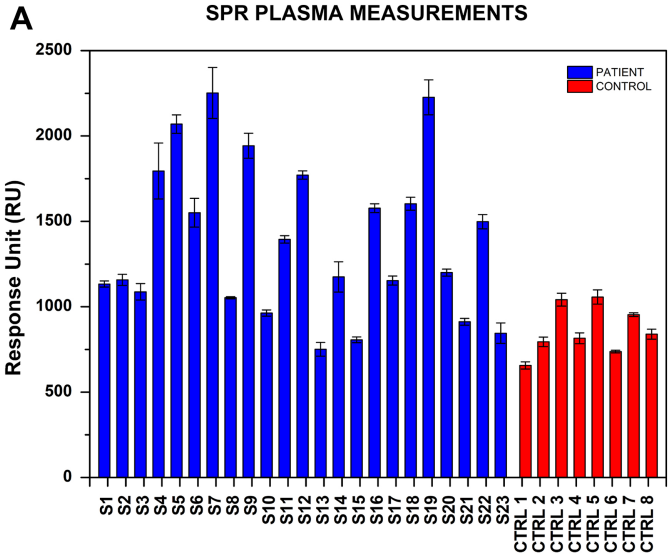

C
Response Unit (RU)
[HMGA1b] nM
HMGA1b
Hill fit curve
Model
Hill
Equation
y=Vmax*x^n/(k^n+x^n)
Reduced Chi-Sqr
4,61563
Adj. R-Square
0,99195

Figure 2


A
SPR PLASMA MEASUREMENTS
Response Unit (RU)
2500
2000
1500
1000
500
0
PATIENT
CONTROL
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
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CTRL 1
CTRL 2
CTRL 3
CTRL 4
CTRL 5
CTRL 6
CTRL 7
CTRL 8

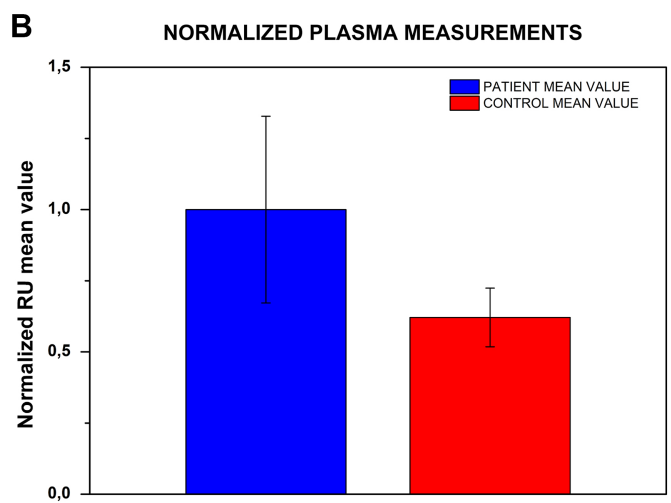
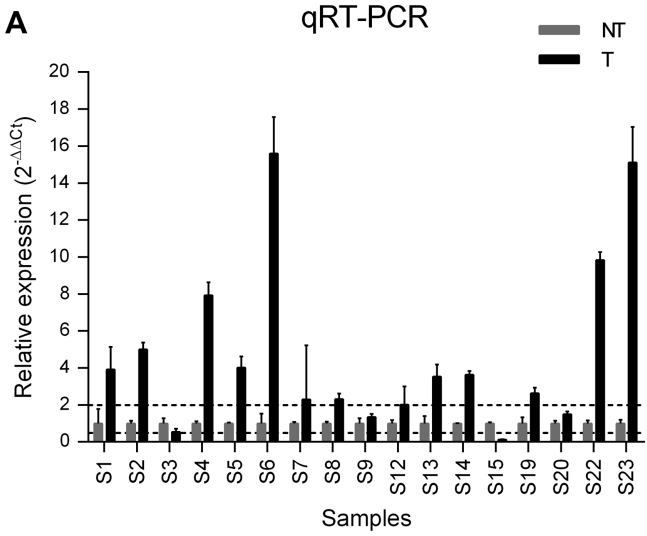

B
NORMALIZED PLASMA MEASUREMENTS
Normalized RU mean value
1,5
1,0
0,5
0,0
PATIENT MEAN VALUE
CONTROL MEAN VALUE

Figure 3


A
qRT-PCR
NT
T
Relative expression ($2^{-\Delta\Delta Ct}$)
0
2
4
6
8
10
12
14
16
18
20
S1
S2
S3
S4
S5
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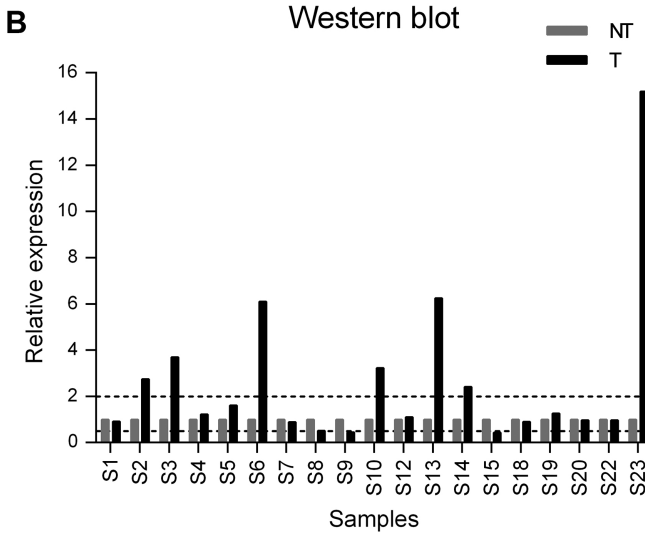
S13
S14
S15
S19
S20
S22
S23
Samples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Western blot
NT
T
Relative expression
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4
6
8
10
12
14
16
S1
S2
S3
S4
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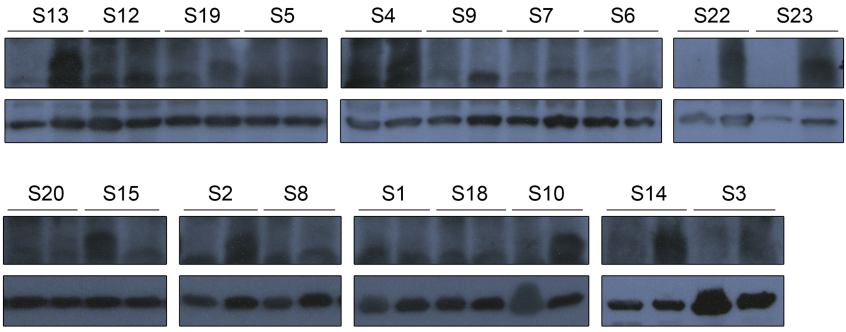
S14
S15
S18
S19
S20
S22
S23
Samples


S13
S12
S19
S5
S4
S9
S7
S6
S22
S23


S20
S15
S2
S8
S1
S18
S10
S14
S3

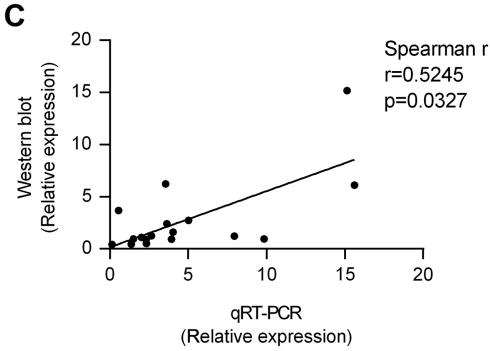
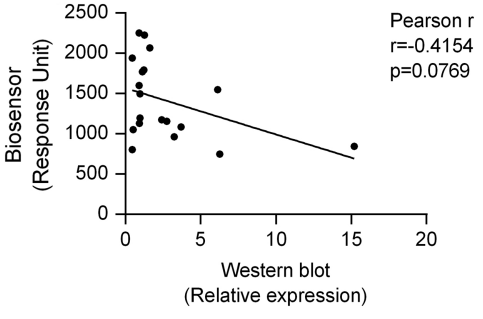

C
Spearman r
r=0.5245
p=0.0327
Western blot
(Relative expression)
0
5
10
15
20
qRT-PCR
(Relative expression)

Figure 4


Biosensor
(Response Unit)
2500
2000
1500
1000
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0
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5
10
15
20
Western blot
(Relative expression)
Pearson r
r=-0.4154
p=0.0769

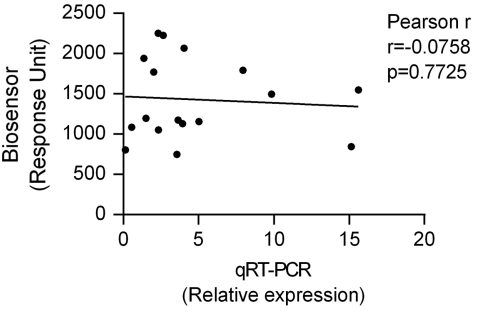
Biosensor
(Response Unit)
2500
2000
1500
1000
500
0
0
5
10
15
20
qRT-PCR
(Relative expression)
Pearson r
r=-0.0758
p=0.7725

Figure 5