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Novel Immunogenic Epitopes in the NaPi-IIb Protein: Production of Monospecific Antibodies Using Synthetic Peptides Outlined on Isoform Specific Regions of the Type IIb Sodium-Dependent Phosphate Transporter (NaPi-IIb)

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Abstract

NaPi-IIb is a multiple passage protein membrane which is primarily responsible for phosphate uptake in the kidney and in the small intestine. Beyond its physiological functions, their involvement with carcinogenesis was initially described in mid-2003, due to its distinct level of expression in normal and tumor cells of the ovary. Although less common than cervical cancer, epithelial ovarian cancer is considered the most lethal gynecologic malignancy, which is mainly due to diagnosis in the advanced stages as a result of the absence of symptoms during the onset of the disease and the lack of tools for early detection. Here, we produce antibodies that are anti-synthetic peptides that are derived from the regions of second extracellular loop of NaPi-IIb, which is a non-overlapping portion of MX35 epitope. These two 15 distinct amino acid residue peptides, designated as Let#1 and Let#2, are engineered in a very thorough way to detect specific sites only in this isoform, thus excluding cross-reactivity with other carriers of the same family. The lack of immunogenicity of small peptides is surpassed by the conjugation with carrier proteins. Using immunochemical methods, we demonstrate that polyclonal antibodies that are mono-specific for

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the Let#1 and Let#2 peptides recognize proteins that express similar amino acid sequences during blood circulation. Additionally, using flow cytometry, we identify NaPi-IIb in NIH:OVCAR-3 cells. The clear identification of two shorter peptides on the extracellular loop of NaPi-IIb, which are far from the monoclonal antibody MX35-recognizing epitopes, adds new promising tools for ovarian cancer follow-up and staging.

Keywords

NaPi2b, Peptides, Antibodies, Ovarian Cancer

1. Introduction

The use of synthetic peptides is a good alternative for antibody production [1] [2]. The choice of molecular domains that simultaneously express functional sites and antigenic epitopes dictate the preference. The significant and identified peptides can be synthesized and strategically modified, and their putative low immunogenicity can be surpassed. The resulting anti-peptide antibodies are used in their native formats or via engineering aimed at improving their epitope-recognizing capacity. Reducing the price of production is also envisaged in the development of anti-peptide antibodies [3]-[5]. After strategic labeling with highly sensitive non-toxic fluorescent compounds, there is increasing use of them in *in vitro* and *in vivo* image diagnosing exams, where cancer, autoimmune and immune deficiency diseases are the targets.

For the development of highly specific antibodies, the selected peptides have to be carefully designed: conserved epitopes and glycosylated sites need to be avoided. In order to prevent potential cross-recognitions, the selected epitope should be variable between homologous proteins. Also, for improving the chances of such epitope being recognized by antibodies, the presence of glycosylation, that camouflages important immunogenic sites, should be considered and avoided. To these ends, molecular alignment techniques are used to compare homologous proteins and determine distinctive variable portions, and thereafter, the analysis of databases provides information about post-translational modifications.

The design of a synthetic peptide should be primarily based on the location of the most immunogenic regions of the protein. *In silico* analysis is widely used to determine the amino acid sequence of these regions. To perform the antigenicity prediction of synthetic molecules, several methods like the ones described by Hopp-Woods [6], Kyte-Doolittle [7] and Goldman EngelmanSteitz [8], are based on the local hydrophobicity and/or hydrophilicity of portions of the molecule. Another method enables to identify the most hydrophilic regions of the protein which are more likely to be extracellular and more accessible for the recognition by antibodies [9] [10]. Besides, a semi-empirical method based on the experimental data of known antigenic determinants can be used: it takes into account the frequency of each amino acid at these antigenic sites, and their probability of presence at the protein surface. Based on this analysis, the immunogenicity of the synthetic sequences can be approximated [11]. Finally, the analysis of molecular flexibility may help improving the fit of the antigen to the antibody sites, which is closely related to the immunogenicity of the peptide sequence [12] [13].

Once the amino acid sequence of the peptide is determined, the ability to stimulate an immune response is investigated. As molecules smaller than 5 kDa are generally not effective for stimulating antibody production, carrier proteins such as KLH (Keyhole Limpet Hemocyanin) and albumins are widely used to enhance immune response. In addition to increasing the size of the hapten and its "visibility" for the immune system, the combination can promote the folding of the peptide to the carrier protein, resulting in a conformation similar to that found in the native protein, thus increasing the chances of the antibody recognizing the target in its original configuration [14].

As a member of the solute carrier family proteins (SLC), sodium-dependent phosphate transporters (NPTs) are membrane proteins that use the electrochemical gradient of sodium ions to conduct inorganic phosphate transport with the kidney and small intestine as the principal organs that maintain the homeostasis of this nutrient [15] [16]. Three types of NPTs (Types I-III) are responsible for this function in vertebrates, which are encoded by the genes of the SLC17 family, SLC20 and SLC34, and they act on distinct molecular mechanisms that depend on the organ in which they are expressed [17].

Type IIb sodium-dependent phosphate transport protein (NaPi-IIb), which is encoded by the SLC34A2 gene, is a membrane glycoprotein that is composed of 690 amino acid residues with molecular weight ranges from 77 - 108 kDa, depending on the degree of glycosylation. In its operational configuration, it presents eight transmembrane domains and four extracellular loops with C and N-terminal moieties found in the cytoplasm [15] [18]-[21].

NaPi-IIb is one of the proteins that is mainly responsible for inorganic phosphate homeostasis [22], and it is widely expressed in the lung, small intestine, kidney, liver, placenta, pancreas, prostate, ovaries, thyroid, uterus, testicle and salivary and mammary glands [15] [23] at the mRNA level. At the protein level, it has been found in the lungs [15] [24]-[26], intestine [27] [28], epididymis [29], mammary glands [25] [26] [30], thyroid, kidneys, uterus [25], salivary glands [31], liver [32], bones and teeth [33]. Two independent studies have shown that mutations in the SLC34A2 gene cause a hereditary pulmonary alveolar microlithiasis, which is characterized by calcium phosphate deposition in the lung alveoli, and it is caused by the loss of carrying capacity of NaPi-IIb phosphate ions into the cells that maintain the homeostatic balance [34] [35]. The mutation of this gene has been hypothesized to be associated with testicular microlithiasis [35].

Beyond its physiological functions, NaPi-IIb has been suggested as a potential epithelial ovarian cancer (EOC) marker [36]. Although less common than cervical cancer, EOC is considered the most lethal gynecologic malignancy, which is mainly due to diagnosis in the advanced stages (FIGO—International Federation of Gynecology and Obstetrics—III and IV) as a result of the absence of symptoms during the onset of the disease and the lack of tools for early detection [37].

The first relationship that was established between NaPi-IIb expression and ovarian cancer occurred in mid-2003. Using serial analysis of gene expression (SAGE) data, as well as public SAGE databases that contained a total of 137 SAGE libraries representing a wide variety of normal and neoplastic tissues, five novel SAGE tags were identified that were specifically expressed in ovarian cancer, and they were named human ovarian cancer-specific transcripts (HOSTs). One of them was NaPi-IIb, which was not previously shown to be expressed in ovarian or other cancers [36].

Interestingly, in 1987, Mattes *et al*. [25] was also looking for ovarian tumor markers and immunized mice with mixtures of fresh ovarian carcinoma cells in an attempt to detect the antigens that were differentially expressed. In this study, it was observed that one of the hybridomas, called MX35, was able to detect an antigen that was differentially expressed in 16 of the 18 ovarian carcinomas tested. However, nothing was known regarding the structure of this antigen. Only after years of study was it possible to characterize NaPi-IIb as an antigen that is recognized by the monoclonal antibody MX35 [21], strengthening the relationship between this protein and ovarian tumor development.

Contrary to what occurs in ovarian tumors, when breast, lung and uterus tissues were assessed by immunohistochemistry, over expression of NaPi-IIb under normal conditions was observed with a decreased expression of this transporter when cells become neoplastic [26], and this perhaps occurs with over expression of others subtypes of NaPi. Using these approaches, many studies were conducted that focused on NaPi-IIb and its relationship with EOC [21] [38]. Preclinical studies in the clinical phase and phase I have pointed to the success in EOC treatments of using anti-NaPi-IIb antibodies that are coupled to anti-tumor drugs, emphasizing the potential of this target in the search for alternatives to aid in the diagnosis, prognosis and treatment of ovarian cancer [39] [40].

Due to the unavailability of sensitive tools and symptoms that appear only in the late stages, epithelial ovarian cancer is a leading cause cancer-related gynecologic deaths [37]. Considering the advancement of molecular biology, specific targets such as NaPi-IIb are crucial for early tracking of diseases and to assist in the prognosis and treatment, and they act as a tool to elucidate the molecular mechanisms that are involved in tumor development. Here, we produced antibodies of anti-synthetic peptides that were derived from the second extracellular loop of the NaPi-IIb protein that contained 188 - 300 amino acids residues in the non-overlapping portion of the MX35 epitope. Delineation of these peptides was performed by Rangel *et al*., which was the first group to establish the relationship between NaPi-IIb and ovarian cancer [36]. The pursuit of these epitopes occurred in a very thorough manner to detect specific sites only on this isoform by excluding cross-reactivity with other carriers of the same family. This feature is relevant for diagnosis and for personalized treatment with reduced side effects that are, for example, provided by blocking others members of the NPTs. Furthermore, isoform specific antibodies are a powerful tool for monitoring NaPi-IIb in healthy and diseased cells. After validation, the produced antibodies were used to assess the possible presence of NaPi-IIb in the serum of patients who suffer from EOC and

in a human serum control. This approach evaluated the possibility of performing diagnosis in a non-invasive manner by using serum samples collected during routine examinations.

2. Material and Methods

2.1. Human Subjects

The use of human serum in this study received approval from the Institutional Ethics Board at the "*Instituto do Câncer do Estado de São Paulo* (*ICESP*), *São Paulo*, *SP*, *Brazil*". All subjects provided written informed consent before undergoing the study procedures. The ovarian cancer was diagnosed via the use of clinical and image assaysusing biopsy collection and histopathological analysis. Women without active diseasesbutwith previously treated tumors of the breast, colon, gastrointestinal tract or endometrium were included as controls (**Table S1**).

2.2. Blood Sample Collection and Processing

Blood was collected via venipuncture, which allowed coagulation, and the serum was separated by centrifugation, divided in aliquots, and stored at −80˚C. The serum samples received two identifications, one containing the subject's personal data and detailed clinical and diagnostic results, which were strictly registered in ICESP files until the end of the experiments, and another using encoded numbers. The first identification was used during the subject treatment, and the second was used during the cancer markers investigation.

2.3. Mice and Ethics Statement

High III strain female mice [41], which were 2 months of age and weighed 18 - 22 g, were obtained from the Immunogenetics Laboratory, Butantan Institute, SP, Brazil. All experimental procedures involving the animals were in accordance with the ethical principles of animal research adopted by the Brazilian Society of Animal Science and the National Brazilian Legislation No. 11.794/08. The protocol was approved by the Animal Care and Use Committee of the Butantan Institute (permission No. 1021/13).

2.4. Synthetic Peptides

Immunogenic peptides were rationally designed to generate specific anti-NaPi-IIb antibodies. Comprehensive *in silico* studies were conducted to avoid non-specific and crossed reactions against the aforementioned antibody. Initially, proteins sequences comprising minimal homology with NaPi-IIb were searched using BLAST (Basic Local Alignment Tool, NCBI, NIH). Isoform a of NaPi-IIb (NP_006415.2) was used as the query sequence in *NCBI Reference Sequences Proteins*, (refseq protein) data base, NCBI, NIH, applying an E-value < 1 amongst human proteins exclusively. Then, the amino acid sequences of proteins with any degree of homology to NaPi-IIb were aligned using ClustalW (Bioedit software) [42]. NaPi-IIb sequence was also mapped for hydrophobic domains according to the Goldman EngelmanSteitz scale [8] [43] [44] to determine targetable potential extracellular loops using the TopPred program, setting the analysis cutoffs from 0.6 to 1.4 [45]. Putative antigenic peptide sequences within the extracellular loops of NaPi-IIb using the Abie Pro 3.0 software (Chang Biosceinces), which is bases on the prediction of hydrophilic amino acids residues following the Hopp-Woods e Kyte-Doolittle scales [6] [7]. Potential peptide epitopes with 15 amino acids were defined within the amino acids 188 and 300 of NaPi-IIb. Moreover, we used the IEDB *Analysis Resources* software [46] to run the Kolaskar-Tongaonkar method to search for antigenic determinants [11], the Emini method to predict superficial accessibility [47], the Karplus Schulz method to predict flexibility [13], and the method of Parker to analyze hydrophilicity [48]. Two potential specific immunogenic peptides were defined for further immunization, named Let#1 and Let#2, which sequences are patent-protected (Patent application number: BR 10 2013 018085 8). For comparison, each peptide had a third peptide that was similar in size but dissimilar in amino acid sequence, named Let#3, that was constructed and modeled on another region from this second protein loop.

Peptides, stored at −20˚C, showed a degree of purity that was greater than 80% and their molecular weight were similar to the theoretical weights available in the databases. For immunization, portions of the synthetic peptides were conjugated with carrier protein "A", which is albumin from mice serum(Sigma-Aldrich, St. Louis, MO, USA) or carrier protein "B", which is ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) using glutaralde-

hyde methodology.

2.5. Mice Immunization Protocols

The High III mice (n = 5/group) were immunized with Let#1 or Let#2 synthetic peptides that were conjugated with protein A or B. The injections of 0.2 mL contained 20 µg of immunogen were given six times at intervals of 2 weeks between the first and fourth immunization and at an interval of 1 month between the fifth and last booster. All of the antigens were mixed with nanostructured silica adjuvant (SBA-15) in a 1:25 proportion (1 part of antigen to 25 parts of SBA-15) [49]. SBA-15 silica was kindly provided by Dr. Osvaldo Augusto Sant'Anna from Butantan Institute, Brazil. The pre-immune serum was collected and analyzed for the eventual presence of the natural anti-peptide antibodies.

2.6. Measurement of Antibodies Using ELISA

Seven days following each immunization, blood was collected by retro-orbital bleeding and incubated for 2 hours at 37˚C. After incubation, the samples were centrifuged at 405×*g*for fifteen minutes at 4˚C, and the supernatant serum was transferred to new tubes and stored at –20˚C. The anti-peptide antibodies were measuredusing an indirect ELISA assay. Briefly, microtiter plates were coated overnight at 4˚C with 1 µg of free or conjugated peptides (coupled to a distinct carrier protein from that used in the immunization) per well in 50 µL of carbonate buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6). Then, they were blocked with 10% nonfat milk in-phosphate-buffered saline (PBS) (8.1 mMsodium phosphate, 1.5 mM potassium phosphate, 137 mMsodium chloride and 2.7 mM potassium chloride, pH 7.2) for 2 h at 37˚C. After incubation, the plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T). The samples of serum collected before (negative control) or after each immunization were diluted 1:500 at intervals of two until 1:128.000 in PBS that contained 0.1% nonfat milk. Aliquots of 100 µL from each dilution were added to the wells and plates that were incubated for 1 h at 37˚C. After washing, 100 µL of peroxidase-conjugated goat anti-mouse IgG (Whole molecule, Sigma-Aldrich, St. Louis, MO, USA) that was diluted 1:2000 in the same buffer was added to the wells and incubated using the same conditions as in the previous step. The peroxidase activity was measured using the o-phenylenediamine (OPD) substrate and read at a wavelength of 490 nm. The highest dilution that produced absorbance values that were at least two times higher than the average pre-immune serum was considered the antibody titer.

2.7. Specificity Analysis of the Anti-Peptide Antibodies via Western Blotting (WB)

The electrophoretic analysis was conducted according to the method previously described by Laemmli (1970) [50]. Conjugated synthetic peptides or the free protein carrier (1 µg) were treated with SDS-PAGE sample buffer under reduction and no-reduction conditions and submitted to electrophoresis in a 10% polyacrylamide gel. To estimate the molecular weight of the components in the samples, the standard molecular mass was used that included prestained protein components between 6 - 180 kDa (BenchMark Pre Stained Protein Ladder, Invitrogen Corp. CA, USA). Some preparations were stained with silver sulfate [51], and others were electroblotted onto nitrocellulose membranes, according the method described by Towbin *et al*. (1979) [52]. These membranes were blocked with a PBS buffer that contained 5% BSA at 37˚C for 2 h, washed with PBS and treated with anti-peptide mouse serum that was diluted 1:64.000 (anti-Let#1) or1:2.000 (anti-Let#2) according to the titer obtained by ELISA or to 1:2000 in the pre-immune serum that was diluted with PBS that contained 0.1% BSA for 1 h at room temperature on a horizontal shaker. After washing three times with PBS that contained 0.05% tween 20, the membranes were incubated with goat anti-mouse IgG that was conjugated to alkaline phosphatase (Whole molecule, Sigma Aldrich, St. Louis, MO, EUA) and diluted 1:7.500 in PBS that contained 0.1% BSA for 1 h at room temperature on a horizontal shaker. The membranes were washed three times with PBS plus 0.05% Tween 20 and transferred to a substrate solution containing 5 mL of AP buffer (100 mM $C_4H_{11}NO_3$, 100 Mm NaCl and 5 mM $MgCl_2$, pH 9.5), 33 µL of NBT (nitro blue tetrazolium, Invitrogen, Eugene, Oregon, EUA) and 16.5 µL of BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Invitrogen, Eugene, Oregon, EUA) for each membrane. Twenty minutes later, the reaction was terminated by washing with distilled water.

2.8. Carrier Protein Response and Cross Reaction

These complementary tests were performed via indirect ELISA-like methodology that was described above. For

all cases, the amount of antigen that was used to sensitize the plates, free carrier or conjugated peptides was fixed at 1 µg/well. The dilution of the anti-peptide antibodies was set at 1:500 for the carrier protein test and 1:100 for the crosslink assays. The next steps remain unchanged.

2.9. Affinity Measurement

The affinity of the anti-peptide antibodies was measured using ELISA as described above with the inclusion of a potassium thiocyanate (KSCN) elution step, according to the methodology previously described by Pullen *et al*. (1986) and Romero-Steiner *et al*. (2005) [53] [54]. After the serum incubation step, 100 µL of KSCN dilutions (0 to 5 M, with intervals of 0.5 M) in PBS were added to each well and incubated for 30 minutes at room temperature. The dilution of the serum used here was set at 1:200 for anti-Let#1 and 1:100 for anti-Let#2. After three washes, a secondary antibody was added, and the following steps were performed as described above. The affinity score was considered at a KSCN concentration that was able to remove 50% of the bound antibodies compared with the respective positive control, *i.e*., the wells with 0 M KSCN.

2.10. Antibody Purification via Affinity Chromatography

The IgG antibodies were purified from pre-immune or hyper-immune sera via affinity chromatography using Hitrap Protein G (GE Healthcare, Pharmacia, United Kingdom), according to the manufacturer's labeling. Then, the preparations were submitted to dialysis (CENTRICON 100, Millipore Corp., Ireland), protein measurement (BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA) and validation by ELISA and WB followingthe methodology described above, except for the dilutions of anti-Let#1 IgG that were used in WB that were set at 1:5000. Finally, they were stored in a freezer at −20˚C until further tests.

2.11. Native NaPi-IIb Recognition

The NIH:OVCAR-3 cells (ATCC®HTB-161™) were grown in 75 cm^2 tissue culture flasks (Corning Inc., New York, USA) containing RPMI 1640 medium(Roswell Park Memorial Institute—Gibco, Invitrogen Corp., CA, USA) that was supplemented with 10% fetal bovine serum (FBS—Cultilab, SP, Brasil) and 1% of penicillin/streptomycin (Gibco, Invitrogen Corp., CA, USA), and they were maintained at 37˚C in a 5% CO_2air atmosphere.

The total number of viable cells was determined by counting in a Neubauer chamber in the presence of Trypan blue, and the concentrations were adjusted to 1×10^6 cells/25 µL in flow cytometry (FCM) buffer (1% BSA and 0.01% sodium azide in PBS) per well. Flow cytometric analysis was performed on the NIH:OVCAR-3 cell suspension that was incubated with previously titrated anti-peptide antibodies, the pre-immune serum or a commercial antibody (polyclonal anti-SLC34A2 antibody produced in rabbit, Abcam®, Cambridge, MA, USA) that were diluted 1:200 for 30 min at 4˚C. Intracellular staining was performed to detect the C-terminal portion of the NaPi-IIbprotein via commercial antibody, and this acted as a positive control. Pre-fixed cells were permeabilized via incubation in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 6 min at room temperature (RT), followed by staining with anti-SLC34A2commercial antibody that was diluted 1:200 for 40 minutes at RT. After three washes, the secondary conjugated FITC anti-mouse or anti-rabbit IgG antibody (Sigma-Aldrich, St. Louis, MO, USA), which was diluted 1:100 in FCM buffer, was added and maintained for 30 minutes at 4˚C (not permeabilized cells) or 40 minutes at RT (permeabilized cells).

Following the cell staining and washing, the samples were fixed with 1% buffered paraformaldehyde (400 µL) prior to analysis by flow cytometry (Canto II - Becton Dickinson, San Jose, CA, USA). The results were expressed as the percentage of the marked cells.

2.12. Patient Serum Analysis

Using the conventional indirect ELISA aforementioned, the presence of the NaPi-IIb protein was evaluated in human serum. Plate wells were individually coated with human serum from ovarian cancer patients or controls that were previously diluted 1:1 in PBS and maintained overnight at 4˚C. Each assay was performed in duplicates. The detection antibodies, anti-Let#1 and anti-Let#2, and the commercial anti-NaPi-IIb antibody used as the positive control, were added at a dilution of 1:100 in PBS that contained 0.1% non-fat milk. The next steps remain unchanged.

2.13. Statistical Analysis

The data were expressed as the mean ± standard error and analyzed statistically using the GraphPad Prism Software (version 5.1 for Windows) (San Diego, USA). The comparisons of more than two groups with one variable were performed using the One-Way ANOVA test, and multiple comparisons were performed by Tukey HSD post-hoc tests. For comparison of two or more variables between more than two groups, the Two-Way ANOVA test was used, followed by Bonferroni post-tests. Student's t-test was used for comparisons between two groups. Statistical correlations were performed using Pearson's correlation test, and for all of the tests, p values of <0.05 were considered significant.

3. Results

3.1. Conjugated Synthetic Peptides Can Induce Specific Antibodies

Free peptides are not effective for validating antibody titers using ELISA (**Figure 1(a)** and **Figure 1(d)**); thus, peptides that were conjugated with different carrier proteins that were used for immunization were used for this purpose. The Let#1 and Let#2 peptides that were conjugated with Acarrier protein (mice albumin) elicited significant IgG antibodies responses to both peptides (**Figure 1(b)**) but not to the carrier protein (**Figure 1(c)**). However, when the peptides were conjugated to the B carrierprotein, ovalbumin (OVA), antibodies to the peptides (**Figure 1(e)**) and to the B carrier protein (**Figure 1(f)**) were produced. Indeed, the anti-peptide antibody titers elicited by A carrier protein conjugated-peptides were significantly higher compared with the anti-peptide

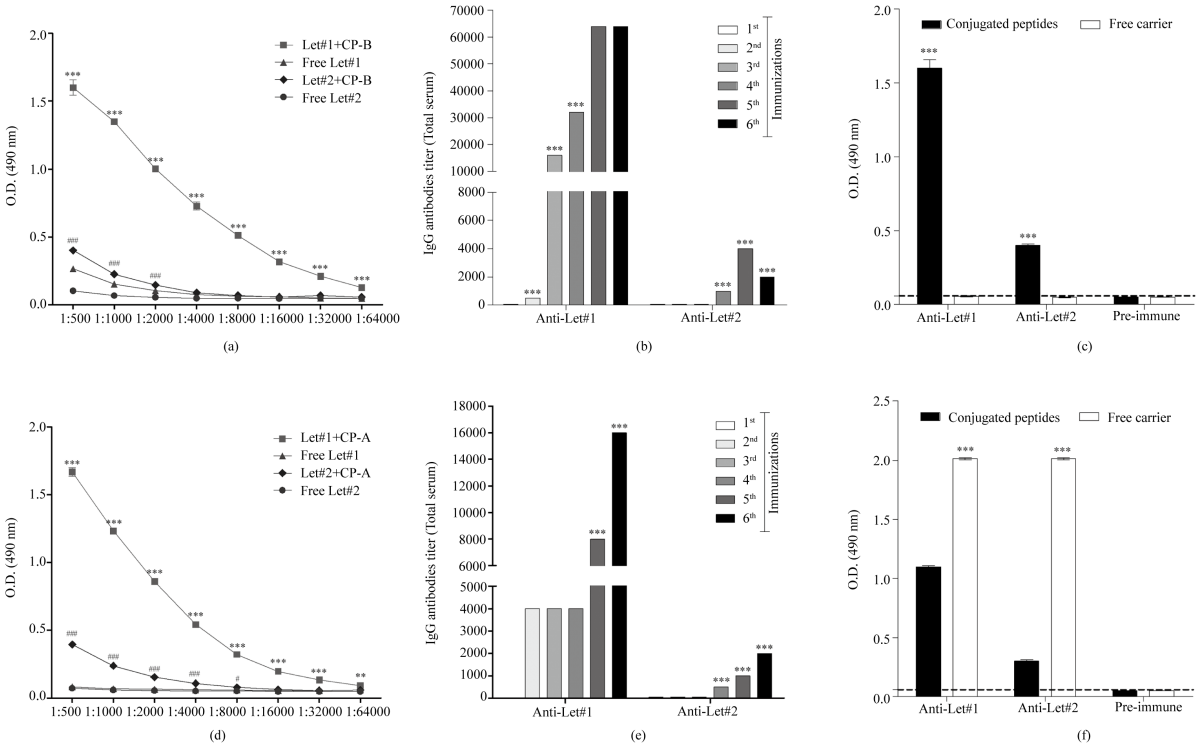

Figure 1. Conjugated Let#1 and Let#2 peptides can induce specific antibodies response. High III mice were subcutaneously immunized with 20 µg of free or conjugated peptides. After each booster, blood samples were collected for analyses via indirect ELISA. For validation of the antibodies that used free or conjugated peptides ((a) and (d)), titration of the anti-peptide antibodies that were produced with immunogen were conjugated with either carrier protein A (CP-A) or CP-B, respectively ((b) and (e)), and crosslinked with the carrier protein that was used for the immunization ((c) and (f)). The data are represented by the mean ± standard error of the duplicates. Significant differences between the mean values obtained for the conjugated or free peptide: * for anti-Let#1 and # for anti-Let#2, titer along the immunizations and conjugated peptides versus the free carrier protein. The titers are expressed as the end point. Comparison included a One-Way ANOVA followed by Tukey's post-tests ((b) and (e)) or a Two-Way ANOVA followed by Bonferroni's post-tests ((a), (c), (d) and (f)). Representative test of three independents experiments. (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$.

antibody titers elicited by the B protein conjugated-peptides, and the antibody titers began to appear after the second immunization and increased until reaching a plateau (**Figure 1(b)**). The free-carrier protein peptides were unable to elicit detectable antibody production (data not shown).

3.2. Cross Linking and Affinity Measurements

The high anti-Let#1 and anti-Let#2 antibodies titer in addition to the lack of anti-carrier protein antibodies it was the relevant features for choice of the serum from mice immunized with carrier protein A-conjugated peptides in the following evaluations. From here, the antibodies were validated with immunogens conjugated to B carrier protein, which is different from that used for immunization, to assess the specific anti-peptide response or with immunogens conjugated to A carrier protein to assess the production of antibody against virtual epitopes.

First, sera from sixty immunizations were used to evaluate the crosslinking between both antibodies with different peptides, including the negative control Let#3 peptide. Here, we demonstrated that there were no cross-reactions among the conjugated Let#1, Let#2 or Let#3 peptides by the anti-Let#1 (**Figure 2(a)**) or anti-Let#2 (**Figure 2(c)**) antibodies. Furthermore, there was no formation of virtual epitopes in the conjugated Let#1, as evidenced by the fact that there was no difference between the recognition of the A-conjugated peptides used for the immunizations or the B-conjugated peptides used for validation. The opposite was observed for peptide 2.

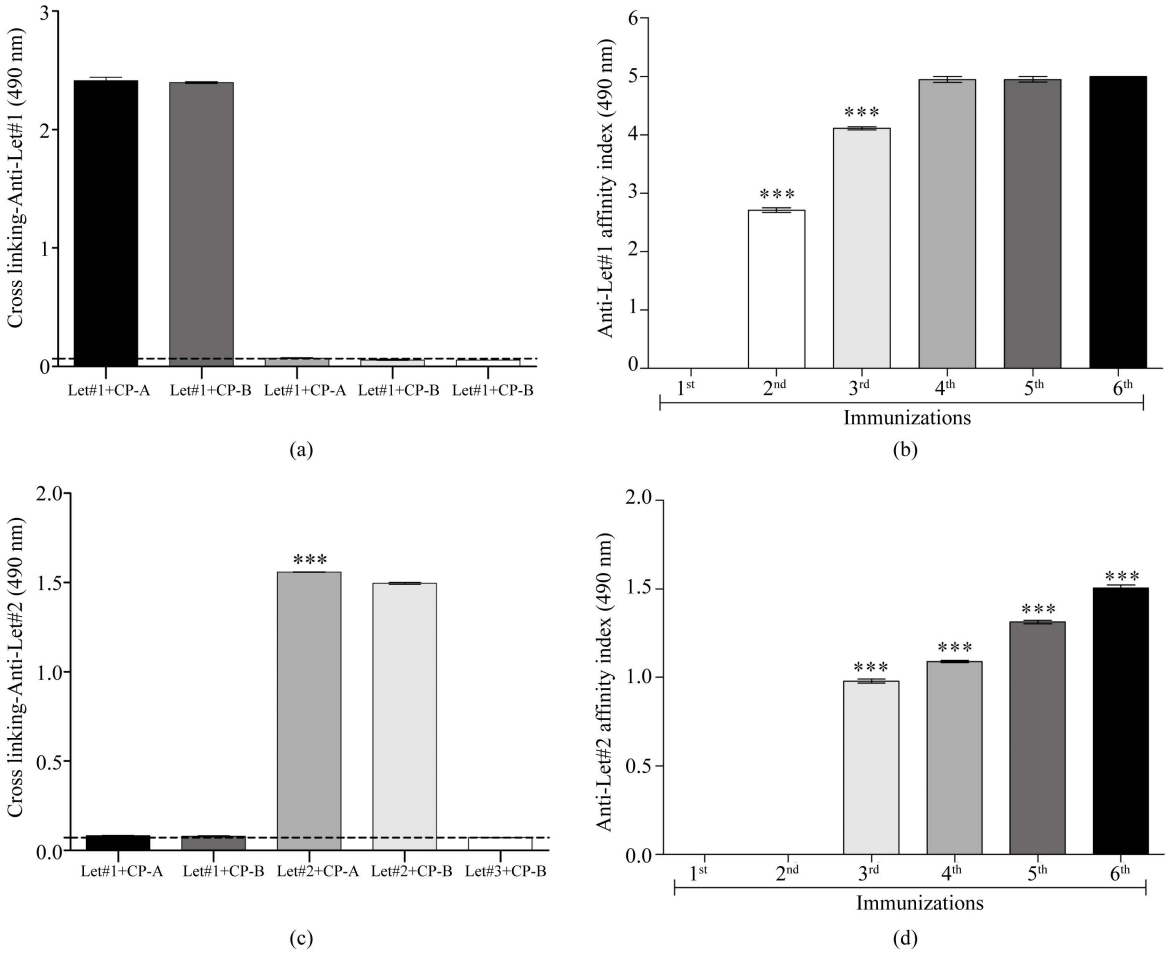

Figure 2. Cross linking and affinity index of the anti-peptide antibodies. Sera from mice immunized with the A-conjugated peptides were selected for these complementary assays. Cross linking of anti-Let#1 (a) or anti-Let#2 (c) antibodies with all peptides conjugated with the A or B carrier protein was conducted, and anti-Let#1 (b) and anti-Let#2 (d) affinity measurements were performed. B carrier protein conjugated-Let#3 peptide was used as a negative control.Data are represented by the mean ± standard error of the duplicates and compared using a One-Way ANOVA followed by Tukey's post-tests. Data are representative of three independent experiments. (***) $p < 0.001$.

Indeed, sera from all immunizations were the antibody sources for the affinity tests. In the affinity assays, anti-peptide/peptide aggregates were treated with different concentrations of the chaotropic agent, KSCN. The following affinity index evaluation criteria was used: the concentration of the chaotropic agent that was sufficient to disrupt 50% of the preformed anti-peptide/peptides, as indicated by the ELISA assaying non-disrupted aggregates. The affinity index was over 5for anti-Let#1 (**Figure 2(b)**) and below 2for the anti-Let#2 (**Figure 2(d)**) antibodies with no significant differences in the affinities of the antibodies produced after the fourth immunization for anti-Let#1 (**Figure 2(b)**). However, there was a slight increase over the immunizations for the anti-Let#2 antibodies (**Figure 2(d)**). Based on the higher affinity values detected after the sixth immunization, these antibodies were selected for further studies.

3.3. Electrophoretic Profiles of Conjugated Peptides and the Specificity of Antibodies via Western Blotting

The protein-conjugated peptides, Let#1 and Let#2, or the free A carrier protein showed electrophoretic profiles with a majority of the bands ranging from 115 kDa to 64 kDa under non-reducing (**Figure 3(a)**) or reducing (**Figure 3(b)**) conditions after submission to SDS-PAGE. With the free carrier or B-conjugated peptides, the relative mass of the bands were approximately 37 kDa to 49 kDa under both conditions. However, these profiles are similar but not identical under non-reducing and reducing conditions. Using Western blotting, these antibodies showed a high specificity and no cross linking with other antigens under the non-reducing conditions: anti-Let#1 (**Figure 3(c)**) and anti-Let#2 (**Figure 3(e)**) and under reducing conditions: anti-Let#1 (**Figure 3(d)**) and anti-Let#2 (**Figure 3(f)**). Additionally, there was no band for the pre-immune serum for both of these conditions (**Figure 3(g)** and **Figure 3(h)**). B carrier protein conjugated-Let#3 peptide was used as a negative control.

3.4. Recognition of Let#1 and Let#2 Like-Peptides on the Native NaPi-IIb Protein Expressed by NIH:OVCAR-3 Cells by Anti-Let#1 and Let#2 Antibodies

The human ovarian NIH:OVCAR-3 cell permeation procedures were not passed, and they were incubated withanti-Let#1 or Let#2 antibodies from the sixth immunization. The cells treated with anti-peptides antibodies, commercial anti-NaPi-IIb antibody and the pre-immune serum (negative control) were subjected to flow cytometry analysis. According to **Figure 4(a)**, we observed a significantly higher specific recognition of the NIH:OVCAR-3 component that expressed the Let#1 and Let#2-like antigenic epitopes of the anti-peptide antibodies compared with the pre-immune serum, whereas the commercial anti-NaPi-IIb antibody recognized any NIH:OVCAR-3 component. In contrast, when the NIH:OVCAR-3 cells were subjected to permeabilization with Triton X-100 before the analysis, as expected, the commercial antibody recognized 80% of the cells (**Figure 4(b)**).

3.5. Detection of the NaPi-IIb Protein in Human Serum by Anti-Let#1 and Let#2 Antibodies

Preliminary tests with thirty-nine human sera samples with anti-peptides antibodies in total serum reveled recognition of Let#1 and Let#2 like-peptides in all of them, while any epitope was detected by pre-immune sera (**Figure 5**). On the other hand, significant statistical differences were observed between samples when revealed by both antibodies (**Table S2**).

After affinity purification, anti-Let#1, anti-Let#2 or pre-immune IgG antibodies were validated using ELISA and WB and used to analyze the thirty-nine human sera samples. Again, the anti-peptide antibodies showed no cross linking with the other conjugated immunogens, according to ELISA (**Figure 6(a)** and **Figure 6(b)**) and WB, for the anti-Let#1 (**Figure 6(e)**), anti-Let#2 (**Figure 6(f)**) or pre-immune IgG (**Figure 6(g)**). Similar to that observed for the total serum, the purified anti-Let#2 IgG showed a significant production of anti-virtual epitope antibodies, as evidenced by the enhanced recognition of carrier protein A-conjugated peptides (**Figure 6(b)**). By using SDS-PAGE under non-reducing and reducing conditions, the purified antibodies showed purity and a satisfactory stability via silver stain (**Figure 6(c)**) or when revealed using anti-IgG antibodies in the WB (**Figure 6(d)**).

Using purified IgG anti-peptide antibodies as the detection tool and under similar assays conditions, the Let#1- and Let#2-like antigenic epitopes were detected, even with different concentrations in all of the assayed sera. Again, there was no recognition of any antigen present in these sera samples when measured with pre-

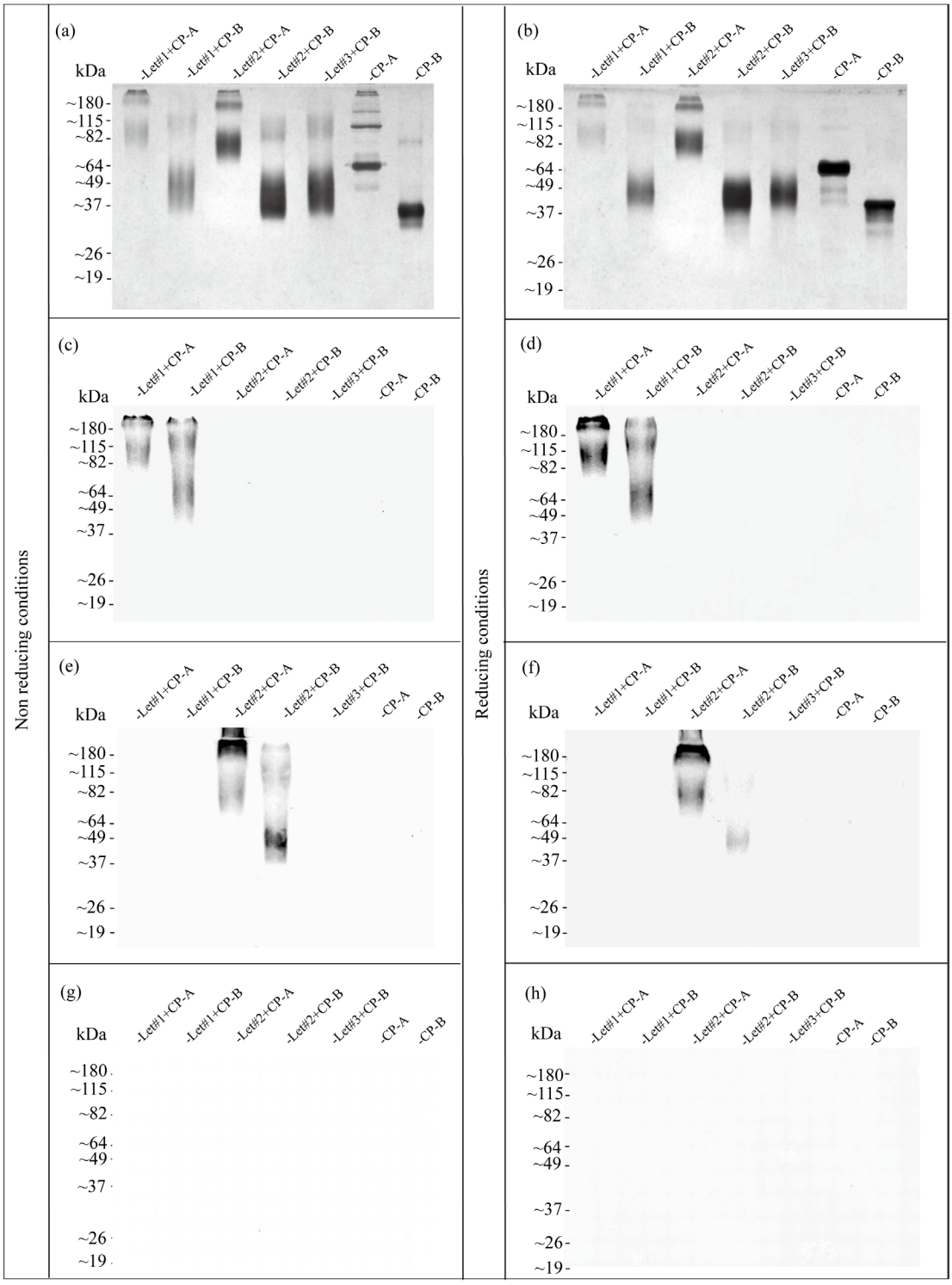

Figure 3. Electrophoretic peptide profile and antibody specificity. The electrophoretic profiles of synthetic peptides that were conjugated to carrier protein (CP) A, CP-B or to the free carriers proteins were observed by SDS-PAGE (upper gel 5% and lower gel 10%), and the specificity of the anti-peptide antibodies was assessed by Western blotting (WB). The peptides were treated with non-reducing ((a), (c), (e) and (g)) and reducing ((b), (d), (f) and (h)) buffer and stained with silver ((a) and (b)) or transferred to a nitrocellulose membrane and revealed with anti-Let#1 ((c) and (d)) or anti-Let#2 ((e) and (f)) antibodies or pre-immune sera ((g) and (h)). B carrier protein conjugated-Let#3 peptide was used as a negative control. The data are representative of three independent experiments.

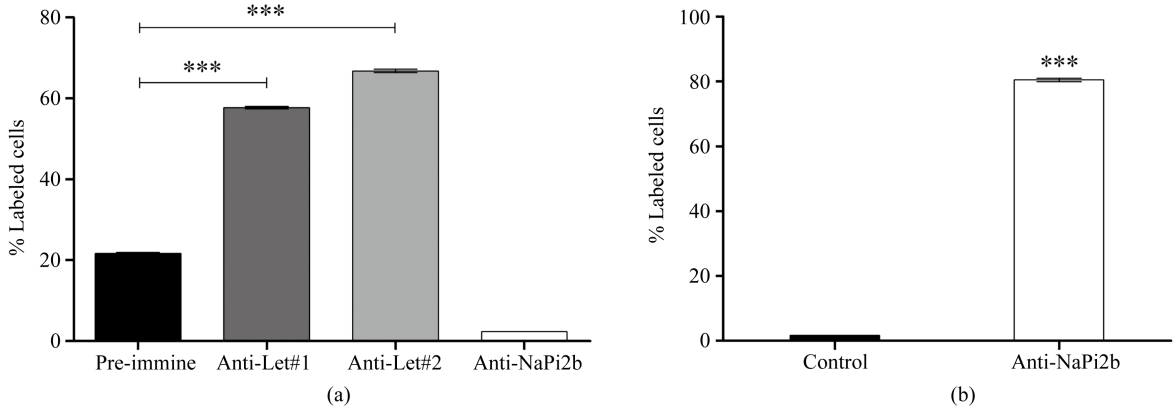

Figure 4. Native NaPi-IIb recognition via flow cytometry. NIH:OVCAR-3 cells, which were derived from human ovarian carcinoma, that did not undergo the permeabilization process (a) were incubated with anti-Let#1, anti-Let#2, pre-immune or commercial anti-NaPi-IIb antibodies. Determination of the commercial anti-NaPi-IIb antibody was evaluated in the cells subjected to permeabilization (b), and the cells treated only with the secondary antibody were the control. The data are represented as the mean ± standard error of duplicates. The data were analyzed statistically by One-Way ANOVA followed by Tukey's post-tests. (*) Significant difference between the pre-immune serum (a) or control with secondary antibody (b). The data are representative of three independent experiments. (***) $p < 0.001$.

immune IgG, indicating a specific recognition of anti-peptide antibodies. However, we observed no significant difference between the mean values of the ovarian cancer patients and the patients who were treated for breast cancer when revealed with the anti-Let#1 (**Figure 7(a)**) or anti-Let#2 antibodies (**Figure 7(b)**). No difference was found when the ovarian cancer patients were compared with all of the other subjects (**Figure 7(c)** and **Figure 7(d)**), as well as when the tumor stage was also considered (**Figure 7(e)** and **Figure 7(f)**). Additionally, the statistical correlations conducted by the Pearson test showed no significant correlation between the evaluated parameters. No interference in the results was found when the anti-peptide antibodies were used together. Positive controls with synthetic peptides were included in all of the assays (data not shown).

To assess the recognition of the samples using a commercial anti-NaPi-IIb antibody, six randomly selected samples were subjected to analysis. Here, we demonstrated that the commercial antibody does not recognize any antigen present in the sera tested with an optical density that was similar to that observed when using the pre-immune sera, which were both below the range of the experiment cutter (0.65) (**Figure 8**). This result suggests that only portions of the NaPi-IIb protein are released in serum, and these portions are possibly located on extracellular loops of the protein, which are the regions encompassing the Let#1 and Let#2 antigens. The C-terminal portion that is recognized by the anti-NaPi-IIb commercial antibody possibly remains within the cell.

4. Discussion

The difficulty of detecting cancer in its early stages leads to its uncontrollable spreading outside of the original organ, triggering poor prognosis, and EOC is not different. EOC is a leading cause of cancer-related gynecologic deaths, causing approximately 15,000 deaths annually in the United States [37]. Due to the lack of symptoms in the early stages and specific markers, only approximately 20% of EOC cases are randomly diagnosed before spreading [55]. Therefore, there is an urgent need for effective alternatives to monitor for this cancer.

Antibodies are powerful tools for the detection of molecules, identification of structural and functional abnormalities, and they can serve as vehicles for transporting toxic agents to target cells. Based on these properties, antibodies that are specific for cancer cells or their products are widely used as auxiliary tools in the diagnosis and treatment of various types of cancer [25] [56] [57].

Antibody production involves the appropriate choice of immunogens, appropriate test animals, standardization of immunization schemes, detection and quantification of the antibodies that are produced, and proper selection of sources for which the antibodies are directed. Once obtained and standardized at the research laboratory level, antibodies have to undergo clinical trials before being transferred to the medical routine.

Our research, as described in previous reports, aims to develop specific antibodies for regions of the NaPi-IIb protein that are normally expressed in certain normal cells but in different amounts in some tumors, such as ova-

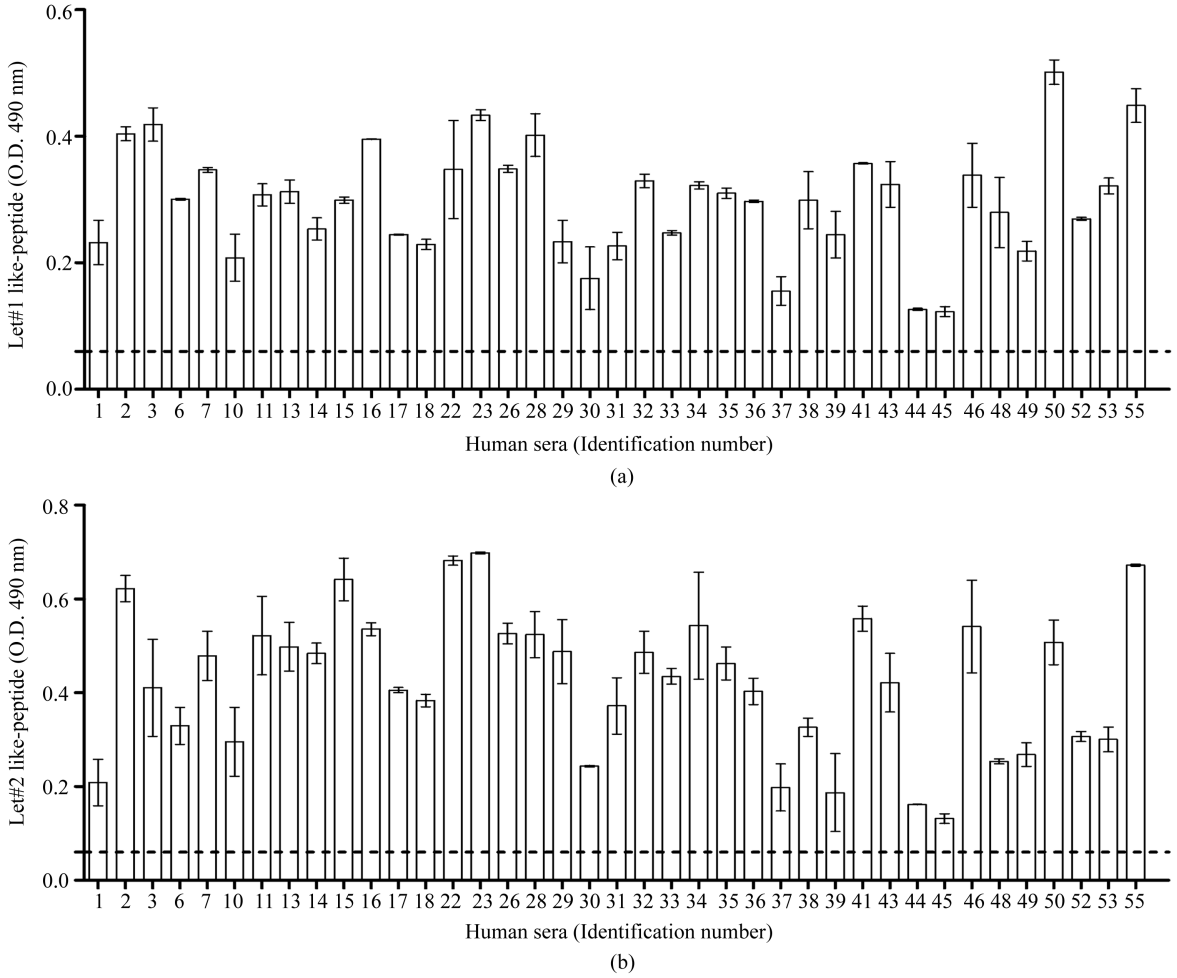

Figure 5. Evaluation of Let#1 and Let#2 like-peptides in human serum samples by anti-peptide antibodies in whole sera. By indirect ELISA, Let#1 and Let#2-like epitopes were evaluated in thirty-nine human sera by anti-Let#1 or anti-Let#2 antibodies. Cutter bar represent the average recognition of pre-immune sera (0.065). Data are represented with a mean ± standard error of duplicates. Analyzed statistically by One-Way ANOVA followed by Tukey's post-test. Representative assay of three independents experiments.

rian carcinoma [36] [58] [59]. Therefore, two peptides, Let#1 and Let#2 are designed using a 188-300 amino acid sequence of the second extracellular loop of NaPi-IIb as a model. Each peptide contains 15 different amino acid residues, which are each separated by 15 amino acid residues. Significant molecular distances separate the MX35 amino acid sequence from the constructed peptide sequences: 17 amino acid residues for Let#1 and 47 amino acid residues for Let#2 [21]. To overcome the lower immunogenicity exhibited by low molecular peptides [60] [61], appropriated protein carriers are selected to conjugate them. The property used to identify the A carrier protein in this work is a sufficient molecular size to accommodate the exposed various peptides, which are processed and recognized by antigen-presenting cells (APC) but not by T or B lymphocytes. In fact, the A carrier-peptides, Let#1 and Let#2, induce antibody populations that are essentially directed to the peptides but not to the carriers.

To optimize the humoral immune response to the peptides, animal species such as mice with high antibody response-selected strains are also used. High III mice are a genetically selected strain that is well-known for its superior capacity to produce circulating antibodies, and this is preferable [41].

Standardized immunochemical methods and the cellular characterization of the antibodies are also applied to detect and quantify the produced antibodies. These combined strategies allow us to develop specific antibodies for the synthetic peptides, Let#1 and Let#2. Anti-peptide antibodies that are titrated using the conventional Enzyme-Linked Immunosorbent Assay (ELISA) indicate that both peptides that are once linked to the appro-

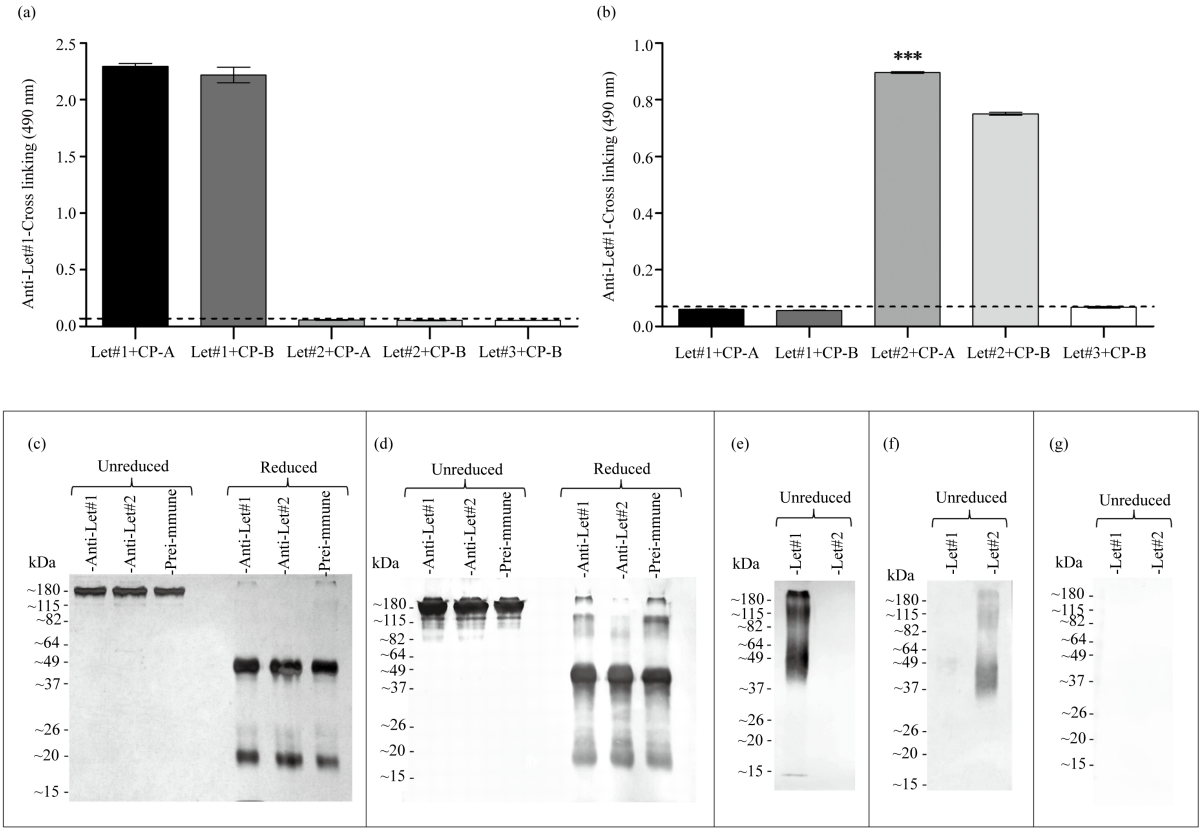

Figure 6. Validation of the purified antibodies. The anti-Let#1, anti-Let#2 or pre-immune purified IgG antibodies were validated by indirect ELISA and WB. Cross linking was conducted for the anti-Let#1 (A and E), anti-Let#2 (B and F) or pre-immune (G) antibodies with peptides that were conjugated with the A or B carrier proteins by ELISA or WB. The electrophoretic profile of the purified antibodies was conducted using a silver stain (C) or by WB that was revealed with anti-mouse IgG (D). The data are represented with the mean ± standard error of duplicates and compared using a One-Way ANOVA followed by Tukey's post-test. The data are representative of three independent experiments. (***) $p < 0.001$.

priate protein carriers become immunogenic, as revealed by the high titers and the mono-specificity. Additionally, the affinity increases during immunization, and the ability to individually recognize the peptide used for immunization is not recognized in the protein carrier epitopes. The results show that the carrier protein can modulate the production of specific antibodies, where A carrier protein has significant immunogenicity to conjugated peptides, as indicated by the induction of high antibody titers of the conjugated peptides. However, this does not occur against its own antigenic epitopes. In contrast, B carrier protein induced antibody production to its own epitopes, and also induced antibodies to the conjugated peptides, although in lesser amounts. However, in the Let#1 peptide, we observed higher immunogenicity compared with the Let#2 peptide, which was expected because the amino acid compositions of the two peptides are different. The structural differences certainly account for either their intra-cellular processing inside the antigen presenting cells (APCs) or for recognition by the B cell receptors once outside these cells, which are bound to MHC class II molecules.

Therefore, we compared the immune response induced by the synthetic peptides conjugated to the carrier proteins that were immunologically tolerated or immunogenic for High III mice. The mice that were immunized with the synthetic peptides conjugated to a tolerated carrier protein (CP-A) responded as follows: 1) a significant level for the specific anti-peptide antibodies; 2) no detectable antibodies for the free carrier protein; and 3) a small amount of antibodies for the epitopes formed by the virtual hapten-carrier interaction in the anti-Let#2 antibodies. However, when the mice were immunized with peptides conjugated to the immunogenic carrier, high titers of anti-carrier antibodies were detected with lower specific anti-peptide antibodies compared with the previous group.

In addition to the immunogenicity of the antigens, the choice of the immunization protocol was of paramount

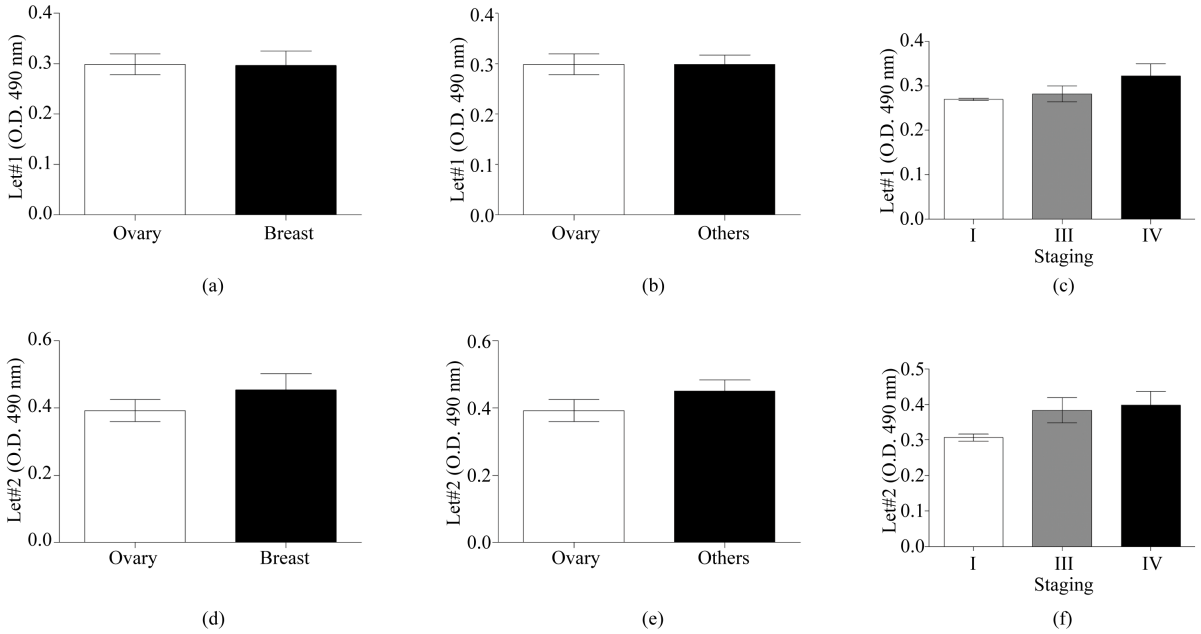

Figure 7. Evaluation of Let#1- and Let#2-like peptides in human serum samples by using purified anti-peptide antibodies. Using indirect ELISA, Let#1- and Let#2-like epitopes were evaluated in thirty-nine human sera samplesby anti-Let#1 ((a), (c) and (e)) or anti-Let#2 ((b), (d) and (f)) antibodies. The comparisons included: ovarian cancer patients versus women who were treated for breast cancer ((a) and (b)), ovarian cancer patients versus other subjects ((c) and (d)) and ovarian cancer stages ((e) and (f)). The data are represented by the mean ± standard error of duplicates, and the data were analyzed statistically by Student's t-test for comparing each of the two groups and using the One-Way ANOVA test followed by Tukey's post-tests for multiple comparisons. We show a representative assay of two independents experiments.

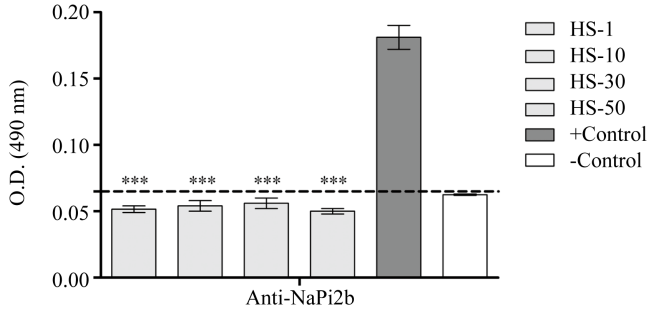

Figure 8. Evaluation of NaPi-IIb in the human serum samples using a commercial antibody. Using indirect ELISA, NaPi-IIb was evaluated in six randomly human sera using a commercial anti-NaPi-IIb antibody. The cutter bar represents the average recognition of the pre-immune sera (0.065). The data are represented by the mean ± standard error of duplicates. The data were analyzed statistically by One-Way ANOVA followed by Tukey's post-tests. There was a significant difference between the sample mean and the recombinant NaPi-IIb (positive control) or the pre-immune sera mean (negative control). This is a representative assay of two independents experiments. (***) $p < 0.001$.

importance. For the production of anti-peptide antibodies, it is recommended to use different experimental groups to overcome drawbacks, such as the use of animal antibody producers that are better than others and the use of more than one peptide that is derived from the protein of interest. The aim is to increase the chances of producing a specific anti-target antibody [3]. Additionally, it is important to minimize the chances of antigens that are tolerated by the immune system, which can be administered in such a way to induce tolerance rather than an effective immune response. Therefore, besides the two different NaPi-IIb derivative peptides, we used three different animal species: White New Zealand rabbits (WNZ), BALB/c and High III mice, and only the lat-

ter species was efficient. As expected, in the High III mice, higher titers of anti-peptide antibodies were obtained. The immunization protocol we used with these animals was different, with intervals of at least two weeks between the immunizations and fewer boosters, which is similar to protocols used in other studies [62]-[64]. However, it cannot be concluded that the best antibody titers obtained in this strain resulted from the immunization protocol used because these mice are intrinsically the best antibody producers, and unlike the rabbits and BALB/c mice, the adjuvant used was SBA-15 silica instead of Marcol Montanide. Therefore, the idea of producing anti-peptide antibodies in rabbits and BALB/c mice using the validated protocol with the High III mice cannot be ruled out.

Although the antibody response was obtained for both immunogens in the High III mice, the high anti-Let#1 antibody titer compared with the anti-Let#2 antibody titer and the higher affinity may be justified by testing the immunogenicity prediction (POPI 2.0) [65], which indicates this peptide is more efficient as an immunogen with potential epitopes that are recognized by $CD4^+$ T cell receptors. Furthermore, these antibodies were quite specific and did not react in a cross-evaluation with other antigens, suggesting that the method is efficient for validating these antibodies as potential identifiers of the native NaPi-IIb protein.

Finally, the developed antibodies were then validated as identifiers of the native NaPi-IIb protein that is expressed on the surface of immortalized ovarian carcinoma cells and in serum of human patients and controls. Using flow cytometry, the data demonstrate the presence of a native NIH:OVCAR-3 cell-surface component that expresses epitopes that are similar to the Let#1 and Let#2 synthetic peptides, and the intracellular presence of epitopes was recognized by the commercial antibody. The antibody titer induced by Let#1+CP-A is much higher than that of Let#2+CP-A. However, in the native protein anchored to the cell surface, the Let#1 epitope appears to be less accessible, since there is less recognized by anti-Let#1 antibodies as compared with the anti-Let#2 antibodies.

NaPi-IIb is widely expressed in several organs of healthy subjects but have abnormal expression in some types of cancer. The increase of the ADAMs family proteins in some tumors [66] [67] that are endowed with the capacity to cleave extracellular domains of transmembrane proteins, supports our hypothesis of the possible release of distinctive levels of NaPi-IIb portions in serum of women with EOC, when compared with healthy subjects. Here, our results point that in sera of women with confirmed ovarian cancer and in sera of healthy subjects used as controls, Let#1 and Let#2-like peptides were found, although in different concentrations, as expected. However, when the obtained data are grouped and the means values of ovarian cancer and controls were compared with each other, there was no statistical difference between them. In addition, the statistic correlations conducted by Pearson's test showed no significant correlation between the evaluated parameters. Thus, it was not possible to identify the ovarian cancer patients using sera samples and anti-peptide antibodies as identifiers of NaPi-IIb protein. In contrast, the detection of any epitope in human sera via the commercial anti-NaPi-IIb antibody can be justified by the distinct localizations of intracellular antigenic epitopes that are exposed along the C-terminal NaPi-IIb molecule domains, instead of the anti-peptide antibodies that are directed toward the Let#1 and Let#2-like antigenic epitopes which are attached to extracellular molecule domains. This results is compatible with the data obtained by flow cytometry in which in intact NIH:OVCAR-3 cells and NaPi-IIb epitopes were detected only by anti-Let#1 and Let#2-like peptides but not by the commercial antibodies. However, when the NIH:OVCAR-3 cells were submitted to the permeation procedures, the NaPi-IIb epitopes were detected by the commercial antibody.

In addition to the difficulty with working with human samples because of the high variations between subjects, a distinct treatment must be considered for use with each sample (see **Table S1**). The NaPi-IIb level may change during the treatment, making it impossible to establish correlations when the women that were selected for the study were at different stages of treatment when the samples were collected. Healthy women and women with newly diagnosed ovarian cancer that have not been subjected to any anti-tumor treatment are better groups to determine the relationship with the level of NaPi-IIb that is released into the circulation. Other experiments are necessary to confirm that this epitope is NaPi-IIb and to try to determine the different level of released NaPi-IIb in normal and tumor serum samples using another approach. However, the absence of any epitope recognition by the pre-immune serum confines the specificity to the used antibodies.

Ultimately, by understanding that available anti-NaPi-IIb antibodies, MX35 [25], humanized MX35 [56] and L2 (2/1) L2 (20/3) and L3 (28/1) [64] recognize the same epitope of the NaPi-IIb protein between the 311 and 340 amino acid residues, it is important to emphasize how few monoclonal antibodies there are against the different domains of the NaPi-IIb protein. Considering the thorough analysis *in silico* required to characterize the

Let#1 and Let#2 peptides, which are portions that are specific to the IIb isoform of this solute carrier, and the successful production of anti-peptide antibodies that recognize the native protein, it is plausible to use them as a potential tool for the specific detection of this protein in healthy and diseased cells. For continuity, the production of monoclonal antibodies against these epitopes is currently in progress in our laboratory.

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Competing Interests

The authors have declared that no competing interests exist.

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Appendix

Table S1. Demographic and Clinical Characteristics of the Participants in the Study.

Primary site	Number in the study	Status	Age	Breed	Menopause	Surgical menopause	Hepatopathy	Histology	Diagnosis data	Stage	Current situation	Pretreatment	If CT, number of rows	Last CT	Previous CA-125 (U/mL)	Sample collection	Current CA-125 (U/mL)
OVARY	1	Elegible	55	White	After	No	No	Serous adenocarcinoma	08/25/2011	III	Under treatment	CT	1	11/30/2012	12210.00	2011/8/12	139.00
OVARY	2	Elegible	37	Pardo	After	Yes	No	Serous Boderline	2008/1/4	IV	Relapse	SG and CT	1	2011/9/12	116.40	2011/9/12	82.20
BREAST	3	Elegible	69	White	After	No	No	Invasive ductal carcinoma	2009/4/5	I	Complete remission	SG, RT and CT	1	12/29/2010	NA	12/13/2011	28.3
OVARY	4	OVARY FAILURE	N/A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12/13/2011	21.9
OVARY	5	OVARY FAILURE	49	Black	Before	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12/13/2011	290
OVARY	6	Elegible	55	Yellow	After	Yes	No	Serous adenocarcinoma	2000/4/8	IV	Under treatment	SG	1	2012/7/10	104.00	12/19/2011	103.80
GASTRO	7	Elegible	80	Pardo	After	No	No	Adenocarcinoma	2009/4/5	I	Complete remission	SG	NA	NA	NA	12/19/2011	11.9
OVARY	8	OVARY FAILURE	56	White	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12/19/2011	22.6
BREAST	9	CONTROL FAILURE	44	Pardo	Before	No	No	Ductal carcinoma	NA	II	Complete remission	SG, RT, CT and HT	NA	2010/10/12	NA	12/19/2011	19.5
OVARY	10	Elegible	59	White	After	Yes	No	Adenocarcinoma	2007/1/1	III	Under treatment	SG and CT	3	12/14/2011	1024.00	12/19/2011	911.40
GASTRO	11	Elegible	73	White	After	No	No	Adenocarcinoma	05/14/2007	IV	Complete remission	SG	1	2209/1/3	NA	12/20/2011	8
OVARY	12	OVARY FAILURE	49	White	Before	No	No	NA	NA	III	Under treatment	CT	1	NA	NA	12/20/2011	NA
OVARY	13	Elegible	62	White	After	Yes	No	Serous adenocarcinoma	2005/1/8	III	Under treatment	SG, RT, CT and HT	>4	08/18/2011	122.00	12/21/2011	264.00
BREAST	14	Elegible	76	White	After	No	No	Ductal carcinoma	2006/1/6	I	Complete remission	SG, RT and HT	NA	NA	NA	12/26/2011	4.6
GASTRO	15	Elegible	69	White	After	No	No	Adenocarcinoma	04/29/2010	II	Complete remission	SG	NA	NA	NA	12/26/2011	17
OVARY	16	Elegible	75	White	After	No	No	Ductal carcinoma	02/13/2010	IV	Under treatment	CT	NA	02/19/2011	299.00	12/27/2011	254.00
GASTRO	17	Elegible	75	White	After	No	No	Adenocarcinoma	2010/1/8	II	Complete remission	SG	NA	NA	NA	2012/10/1	8.8
BREAST	18	Elegible	57	White	After	No	No	Carcinoma Ductal	2010/1/11	I	Complete remission	SG, RT, CT and HT	1	08/31/2010	NA	2012/10/1	16.3
OVARY	19	OVARY FAILURE	40	White	After	No	No	Serous adenocarcinoma	NA	III	Under treatment	SG	NA	01/21/2012	16.9	01/19/2012	17.6
OVARY	20	OVARY FAILURE	56	White	After	Yes	No	Serous adenocarcinoma	2012/9/3	IV	NA	SG and CT	2	12/22/2012	NA	01/19/2012	7.4
BREAST	21	CONTROL FAILURE	47	White	Before	No	No	Ductal	NA	III	Complete remission	SG and HT	NA	NA	NA	01/19/2012	11.4
BREAST	22	Elegible	67	White	After	No	No	Ductal carcinoma	12/29/2008	I	Complete remission	SG, RT, CT and HT	1	2009/4/5	NA	01/23/2012	12.2
BREAST	23	Elegible	54	White	After	No	No	Ductal carcinoma	2011/1/2	I	Complete remission	SG, RT and HT	NA	NA	NA	01/23/2012	15.2
OVARY	24	OVARY FAILURE	73	NA	After	Yes	No	Serous adenocarcinoma	2010/1/4	III	Under treatment	SG and CT	3	01/24/2012	5.1	01/23/2012	4.9
OVARY	25	OVARY FAILURE	77	White	After	No	No	Poorly differentiated carcinoma	2011/11/8	III	Under treatment	NA	NA	2012/6/1	6.5	02/23/2012	6.3
OVARY	26	Elegible	65	White	After	No	No	Adenocarcinoma	05/25/2011	III	Under treatment	CT	NA	2011/1/11	126.00	01/26/2012	72.50

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OVARY	27	OVARY FAILURE	34	White	After	No	No	Mucinous adenocarcinoma	2011/3/10	I	Under treatment	SG and CT	NA	2012/10/1	NA	01/26/2012	18
SARCOMA	28	Elegible	78	White	After	No	No	Splindle cell sarcoma	2010/1/6	I	Complete remission	SG and RT	NA	NA	NA	01/26/2012	9.5
BREAST	29	Elegible	50	White	After	No	No	Ductal carcinoma	11/25/2009	III	Complete remission	NA	NA	05/27/2010	NA	01/26/2012	15.1
BREAST	30	Elegible	78	White	After	No	No	Ductal carcinoma	05/15/2010	I	Complete remission	SG, RT and HT	NA	NA	NA	01/27/2012	11.2
BREAST	31	Elegible	48	White	After	No	No	Ductal carcinoma	09/16/1963	II	Under treatment	SG, RT and HT	NA	NA	NA	01/27/2012	14.1
BREAST	32	Elegible	62	White	After	No	No	Ductal carcinoma	2010/1/6	II	Complete remission	SG,RT and CT	NA	2010/12/12	NA	01/27/2012	22.3
GASTRO	33	Elegible	84	Pardo	After	No	No	Adenocarcinoma	2007/5/4	II	Complete remission	SG	NA	NA	NA	01/27/2012	15.8
GASTRO	34	Elegible	69	White	After	No	No	Adenocarcinoma	01/28/2010	II	Complete remission	SG	NA	NA	NA	01/31/2012	6.1
OVARY	35	Elegible	59	White	After	Yes	No	Serous adenocarcinoma	1952/9/9	IV	Under treatment	CT	1	09/26/2012	3291.00	2012/1/1	3108.00
OVARY	36	Elegible	62	White	After	Yes	No	Serous adenocarcinoma	2009/1/11	III	Under treatment	SG and CT	2	2012/5/1	45.50	2012/2/2	38.80
BREAST	37	Elegible	61	Pardo	After	No	No	Ductal carcinoma	01/13/2009	I	Complete remission	SG	NA	NA	NA	2012/3/2	6.2
BREAST	38	Elegible	69	Pardo	After	No	No	Ductal carcinoma	2010/1/3	II	Complete remission	SG,RT and HT	NA	NA	NA	2012/3/2	14.3
COLON	39	Elegible	54	Black	After	No	No	Adenocarcinoma	2007/5/1	III	Complete remission	SG and CT	NA	2008/1/9	NA	2012/3/2	7.7
ENDOMETRIUM	40	CONTROL FAILURE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2012/6/2	13.7
OVARY	41	Elegible	42	White	After	Yes	No	Papillary adenocarcinoma	08/25/2010	III	Under treatment	SG and CT	2	01/13/2012	77.20	2012/8/2	85.60
OVARY	42	OVARY FAILURE	36	NA	After	No	No	Serous Boderline	01/30/2011	III	NA	SG	NA	NA	NA	2012/8/2	10.6
OVARY	43	Elegible	63	White	After	No	No	Serous adenocarcinoma	11/21/2011	III	NA	CT	1	01/24/2012	248.00	2012/9/2	125.00
OVARY	44	Elegible	47	White	After	Yes	No	Serous adenocarcinoma	11/19/2010	IV	Under treatment	SG and CT	1	2011/4/4	79.00	2012/10/2	76.60
OVARY	45	Elegible	50	White	After	Yes	No	Serous adenocarcinoma	2009/1/7	III	Under treatment	SG and CT	2	09/30/2012	64.80	02/13/2012	68.30
OVARY	46	Elegible	57	White	After	Yes	No	Serous adenocarcinoma	2011/5/9	III	Under treatment	SG and CT	1	2012/7/2	329.00	02/27/2012	170.00
OVARY	47	OVARY FAILURE	71	White	After	Yes	No	Adenocarcinoma	2002/1/1	III	Relapse	SG and CT	2	2009/1/2	65	02/28/2012	34.9
OVARY	48	Elegible	50	White	After	Yes	No	Serous adenocarcinoma	2011/2/3	III	Under treatment	SG and CT	2	01/27/2012	15731.00	2012/1/3	20613.00
OVARY	49	Elegible	54	White	After	Yes	No	Serous adenocarcinoma	04/26/2011	IV	Relapse	CT	NA	2011/1/8	67.90	2012/6/3	85.00
OVARY	50	Elegible	58	White	After	Yes	No	Adenocarcinoma	01/20/2012	IV	Under treatment	CT	1	2012/1/3	2906.00	03/14/2012	2152.00
OVARY	52	Elegible	64	White	After	No	No	Papillary adenocarcinoma	2004/1/1	I	Relapse and under treatment	NA	3	01/27/2012	8200.00	03/15/2012	5790.00
OVARY	53	Elegible	56	White	After	No	No	Adenocarcinoma	02/21/2012	IV	Under treatment	NA	NA	NA	3640.00	03/21/2012	104.00
OVARY	54	OVARY FAILURE	71	NA	After	No	No	Poorly differentiated carcinoma	2011/1/10	III	NA	NA	NA	2012/5/3	221	03/21/2012	24.5
BREAST	55	Elegible	65	Pardo	After	No	No	Invasive ductal carcinoma	04/13/2011	II	NA	SG, RT and CT	1	11/29/2011	NA	03/27/2012	33

*All individuals enrolled for the study are female; Underling rows refers to patients who were excluded of the study due to failure; CT: Chemotherapy; SG: Surgery; RT: Radioteraphy; HT: Hormone teraphy and NA: Not Available.

Table S2. Statistical differences between Let#1 and Let#2 like-peptides concentration in thirty-nine human sera samples.

Anti-peptides antibodies											
Anti-Let#1 antibody						Anti-Let#2 antibody					
Samples	**≠**	**Samples**	**≠**	**Samples**	**≠**	**Samples**	**≠**	**Samples**	**≠**	**Samples**	**≠**
1 vs 2	*	15 vs 44	*	31 vs 55	***	1 vs 2	***	15 vs 39	***	30 vs 41	*
1 vs 3	**	15 vs 45	*	32 vs 37	*	1 vs 11	*	15 vs 44	***	30 vs 46	*
1 vs 16	*	15 vs 50	**	32 vs 44	**	1 vs 15	***	15 vs 45	***	30 vs 55	***
1 vs 23	**	16 vs 18	*	32 vs 45	**	1 vs 16	*	15 vs 48	***	31 vs 55	*
1 vs 28	*	16 vs 29	*	32 vs 50	*	1 vs 22	***	15 vs 49	**	32 vs 39	*
1 vs 50	***	16 vs 30	***	33 vs 50	***	1 vs 23	***	15 vs 52	**	32 vs 44	*
1 vs 55	***	16 vs 31	*	33 vs 55	**	1 vs 26	*	15 vs 53	**	32 vs 45	**
2 vs 10	**	16 vs 37	***	34 vs 37	*	1 vs 28	*	16 vs 30	*	33 vs 45	*
2 vs 18	*	16 vs 44	***	34 vs 44	**	1 vs 34	**	16 vs 37	**	34 vs 37	**
2 vs 29	*	16 vs 45	***	34 vs 45	**	1 vs 41	**	16 vs 39	**	34 vs 39	**
2 vs 30	***	16 vs 49	*	34 vs 50	*	1 vs 46	**	16 vs 44	**	34 vs 44	**
2 vs 31	*	17 vs 23	**	35 vs 44	**	1 vs 50	*	16 vs 45	***	34 vs 45	***
2 vs 37	***	17 vs 50	***	35 vs 45	**	1 vs 55	***	17 vs 23	*	35 vs 44	*
2 vs 44	***	17 vs 55	**	35 vs 50	**	2 vs 6	*	18 vs 22	*	35 vs 45	*
2 vs 45	***	18 vs 23	**	36 vs 44	*	2 vs 10	*	18 vs 23	*	37 vs 41	**
2 vs 49	**	18 vs 28	*	36 vs 45	*	2 vs 30	**	22 vs 30	***	37 vs 46	**
3 vs 10	**	18 vs 50	***	36 vs 50	**	2 vs 37	***	22 vs 31	*	37 vs 50	*
3 vs 14	*	18 vs 55	***	37 vs 41	**	2 vs 38	*	22 vs 37	***	37 vs 55	***
3 vs 17	*	22 vs 30	*	37 vs 43	*	2 vs 39	***	22 vs 38	**	38 vs 55	**
3 vs 18	**	22 vs 37	**	37 vs 46	**	2 vs 44	***	22 vs 39	***	39 vs 41	**
3 vs 29	**	22 vs 44	***	37 vs 50	***	2 vs 45	***	22 vs 44	***	39 vs 46	**
3 vs 30	***	22 vs 45	***	37 vs 53	*	2 vs 48	**	22 vs 45	***	39 vs 50	*
3 vs 31	**	23 vs 29	**	37 vs 55	***	2 vs 49	**	22 vs 48	***	39 vs 55	***
3 vs 33	*	23 vs 30	***	38 vs 44	*	2 vs 52	*	22 vs 49	***	41 vs 44	***
3 vs 37	***	23 vs 31	**	38 vs 45	*	2 vs 53	*	22 vs 52	**	41 vs 45	***
3 vs 39	*	23 vs 33	**	38 vs 50	**	6 vs 15	*	22 vs 53	**	41 vs 48	*
3 vs 44	***	23 vs 37	***	39 vs 50	***	6 vs 22	**	23 vs 30	***	41 vs 49	*
3 vs 45	***	23 vs 39	**	39 vs 55	**	6 vs 23	**	23 vs 31	*	43 vs 45	*
3 vs 49	**	23 vs 44	***	41 vs 44	***	6 vs 55	**	23 vs 36	*	44 vs 46	**
6 vs 44	*	23 vs 45	***	41 vs 45	***	7 vs 39	*	23 vs 37	***	44 vs 50	**
6 vs 45	*	23 vs 49	***	43 vs 44	**	7 vs 44	*	23 vs 38	**	44 vs 55	***
6 vs 50	**	23 vs 52	*	43 vs 45	**	7 vs 45	**	23 vs 39	***	45 vs 46	***
7 vs 30	*	26 vs 30	*	43 vs 50	*	10 vs 15	**	23 vs 44	***	45 vs 50	**

Continued

7 vs 37	**	26 vs 37	**	44 vs 46	**	10 vs 22	***	23 vs 45	***	45 vs 55	***
7 vs 44	***	26 vs 44	***	44 vs 50	***	10 vs 23	***	23 vs 48	***	48 vs 55	***
7 vs 45	***	26 vs 45	***	44 vs 53	**	10 vs 55	**	23 vs 49	***	49 vs 55	***
10 vs 16	**	28 vs 29	*	44 vs 55	***	11 vs 37	*	23 vs 52	***	52 vs 55	**
10 vs 23	***	28 vs 30	***	45 vs 46	***	11 vs 39	**	23 vs 53	***	53 vs 55	**
10 vs 28	**	28 vs 31	*	45 vs 50	***	11 vs 44	**	26 vs 37	*		
10 vs 50	***	28 vs 37	***	45 vs 53	**	11 vs 45	***	26 vs 39	**		
10 vs 55	***	28 vs 44	***	45 vs 55	***	13 vs 37	*	26 vs 44	**		
11 vs 44	*	28 vs 45	***	46 vs 50	*	13 vs 39	*	26 vs 45	***		
11 vs 45	**	28 vs 49	**	48 vs 50	***	13 vs 44	**	28 vs 37	*		
11 vs 50	**	29 vs 50	***	48 vs 55	*	13 vs 45	**	28 vs 39	**		
13 vs 44	**	29 vs 55	***	49 vs 50	***	14 vs 39	*	28 vs 44	**		
13 vs 45	**	30 vs 41	*	49 vs 55	***	14 vs 44	*	28 vs 45	***		
13 vs 50	**	30 vs 46	*	50 vs 52	***	14 vs 45	**	29 vs 39	*		
14 vs 23	*	30 vs 50	***	50 vs 53	*	15 vs 30	***	29 vs 44	*		
14 vs 50	***	30 vs 55	***	52 vs 55	*	15 vs 37	***	29 vs 45	**		
14 vs 55	**	31 vs 50	***			15 vs 38	*	30 vs 34	*		

Here are shown only samples with statistical differences. Analyzed statistically by One-Way ANOVA followed by Tukey's post-test. Representative assay of three independents experiments. (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$.