Model building and quantitative analysis of a tandem immuno-capturing assay as a screening tool for breast cancer

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Abstract. The onset of breast cancer appears to occur, on average, a decade earlier in Mexican women in comparison to American or European women. Early detection and prevention of breast cancer are of crucial importance to increase survival and improve quality of life. Based on the molecular elucidation of critical events leading to breast carcinogenesis, a tandem immuno-capturing blood test was developed as a quantitative population screening assay in view of providing a cost-effective and non-invasive alternative to population screening. Clinical analysis of 63 Mexican women within an age group of 35-70, revealed that Interstron activity increases from 800±65 IU_{JPA} (Interstron Units) in the asymptomatic normal women to 994±100 IU_{JPA} in the symptomatic/benign group, reaching 1289±81 IU_{JPA} in the cancerous group. Accordingly, activity thresholds were established at 800 and 1200 IU_{JPA} respectively, encompassing three risk groups: (i) Healthy Otherwise Normal (<800 IU_{JPA}); (ii) Grey Risk Area (>800 and <1200 IU_{JPA}), and (iii) At Risk group (>1200 IU_{JPA}). Taking into account both baseline and clinical case reports, the Healthy Otherwise Normal group and the At Risk group were mostly homogeneous in nature, comprising a population of normal and cancer patients respectively. The Grey Risk group is heterogeneous, likely reflecting a transitional nature towards a potential early stage of breast disease development. Based on these results, a screening algorithm was developed as the underlining principle for population surveillance encompassing over 30,000 Mexican women. The current screening results have enabled us to objectively prioritize medical attention to approximately 1 in 8 women out of the general population mapped within the At Risk group. Overall, our findings suggest that monitoring Interstron activity units provides a valuable quantitative screening analysis as to selectively streamline the population of women in need of early medical counseling and/or mammography, thereby enhancing both the quality and cost-effectiveness of preventative population surveillance programs targeting breast cancer.

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Introduction

Early detection of breast cancer enables more efficacious treatment and disease management (1). Although current guidelines for breast cancer screening include both breast examination and mammography (2), monthly breast self-examination is currently being reassessed in view of recent findings underscoring its limitations (3). Mammography remains the primary and the most acceptable screening tool (4). Nonetheless, the benefits of screening mammography may be limited among women <50 years of age since such population tend to have higher breast density, making false-positives mammograms more likely (5,6). In Mexican women this presents a dilemma since the average onset of breast cancer is about a decade earlier than the American or European women, consequently general guidelines on mammography would not cover 50% of Mexican women (7,8). From a genetic perspective, the estimated relatively low frequency of mutations on genetic markers such as BRCA1 and BRCA2 restricts the usefulness of genetic testing to <10% of the general population (9-11). Furthermore, adaptation of genetic tests or mammography as a generic screening procedure may encounter serious logistical and economical implications. For instance, according to 2002 census, there are an estimated 33.7 million women currently living in the United States with age ranging between 45 and 64 (U.S. Census, 2002). Assuming a typical minimal cost of $100 per mammogram (National Cancer Institute, 2002), if this population of women had subjected themselves to annual

mammograms, the cost would have exceeded $3 billion. This would have represented approximately half of the actual 2002 reported cost for medical treatment ($6 billion) of breast cancer in the United States (American Cancer Society, 2002). The necessity for efficient disease control and cost reduction entails the articulation of a more effective population surveillance program to promote prevention and early detection of breast cancer.

Amongst all suspected risks factors, estrogen and estrogen-stimulated cell cycle regulators remain the most direct and important determinants in relation to the onset and progression of breast carcinogenesis (12-18). Recent population based studies have revealed strong evidence indicating that sustained use of HRT (Hormone Replacement Therapy) is associated with an increased risk of breast cancer, particularly of invasive lobular tumors hence hampering the efficiency of mammography (18-22). Furthermore, enhanced estrogen-driven mammary epithelial proliferative capacity can be partially explained by lack of expression of functional BRCA1 peptide (23), hence providing a link between life style and environmental factors to genetic risk factors believed to be involved in the early onset of breast cancer. In brief, the identification of quantifiable estrogenic risk factors that are indicative of lifestyle and environmental influences would be an intuitive logistic behind an optimal population surveillance program.

It is within this context that we have introduced the concept of monitoring the molecular onset of breast tumour formation and metastasis through critical estrogen-inter-dependent cellular markers (24). Changes in the distribution and circulating levels of both the Leucine Amino Peptidase (LAPase, EC 3.4.11.1) and the Nucleoside Diphosphate Phosphotransferase (NDP-K, EC 2.7.4.6) in response to sustained cell mediated stimulation of 17ß-Estradiol have been amply documented (24-30). In view of their enzymatic stability, steroid dependency, and crucial role in the immediate early events of cellular proliferation within the cell cycle and cell spreading, NDP-K and LAPase are valuable quantitative cellular predictors of early tumour formation. Using a quantitative first order assay and specific monoclonal antibodies against both NDP-Kinase and LAPase respectively, it was shown that these enzymes were elevated in women affected by breast cancer (24,26-28).

This pioneering work has led to the development of a blood-based tandem immuno-capturing assay for breast cancer screening (29,30). This report summarizes the results from a clinical analysis performed in Mexico with the assistance of the Department of Oncology of the Mexican General Hospital. Apart from confirming the efficacy of the immuno-capturing assay aforementioned as found in previous studies, this clinical study was intended to establish empirical thresholds for a putative population screening algorithm. Cross-matching of the reported pathology classification with that of the estimated plasma activity levels of Interstron activities has led to the definition of three distinctive groups: Healthy Otherwise Normal Women (<800 IU_{JPA}), Grey Risk Area (800 to 1200 IU_{JPA}) and At Risk group (>1200 IU_{JPA}). Based on the established clinical thresholds, a population surveillance algorithm was developed and subsequently deployed to perform a mega field analysis on the general population of Mexican women. The feasibility of adopting the tandem immuno-capturing blood test as a pre-filter for prioritizing medical attention to the At Risk group is discussed in terms of its efficiency and efficacy.

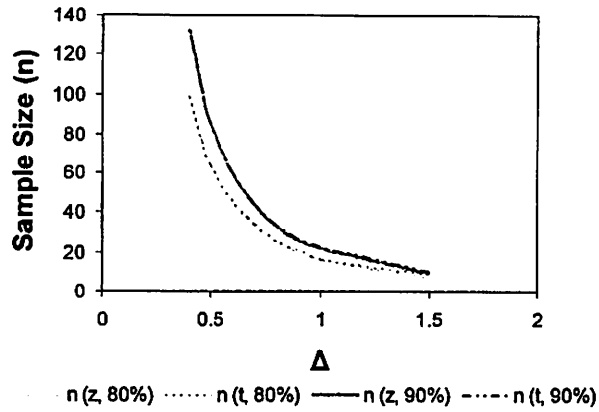


Figure 1. Sample size estimation by z- and t-statistics. This plot is based on the sample size formula presented in Equation 1 and 2. The assumed confidence level is 0.95 (two-tailed) and at a power of 0.80 and 0.90 respectively.

Materials and methods

Study design

Objective setting. Results from two studies are summarized in the Results and Discussion sections. The first study was the clinical trial that established the analysis model for the Interstron/Biodecan assay. The main objective of the study was to quantify the difference between normal and cancerous subjects in terms of Interstron activities. Following the determination of segregation thresholds, a population analysis was subsequently carried out to evaluate the efficiency and efficacy of Interstron/Biodecan as a quantitative screening tool for breast cancer.

Sample size estimation. For clinical analysis the response variable was Interstron activity, which was a continuous parameter. Therefore, z-statistics or t-statistics was expected to be used to evaluate the significance of the difference exhibited between the groups of subjects. Sample size formula from z and t-statistics based on two-tailed tests are shown in Equation 1 and 2 below, where α is the type I error, ß is the type II error, δ denotes the difference in population, and σ, s represent standard deviation. Equation 1: $n= [(Z_{1-\alpha/2} + Z_{1-\beta})/\delta]^2 \times 2\sigma^2$ and Equation 2: $n=[(t_{1-\alpha/2,2(n-1)} + t_{1-\beta,2(n-1)})/\delta]^2 \times 2\sigma^2$

The above sample size formulas can be reduced to a function of effective size Δ, which is the ratio of group difference to the standard deviation (31). In Fig. 1, sample size n was plotted as a function of Δ at a confidence level of 0.95, and power level of 0.80 and 0.90. Our previous clinical data suggested that Δ≈1. Therefore, according to Fig. 1 the minimum sample size is around 16-17 per group at a power of 0.80 and 22-23 at a power of 0.90. The chosen target sample size for the clinical trial was 30 per group, which brings the total expected enrollment to 60. The actual enrollment was 63, with 32 and 31 women in the case and control groups, respectively.

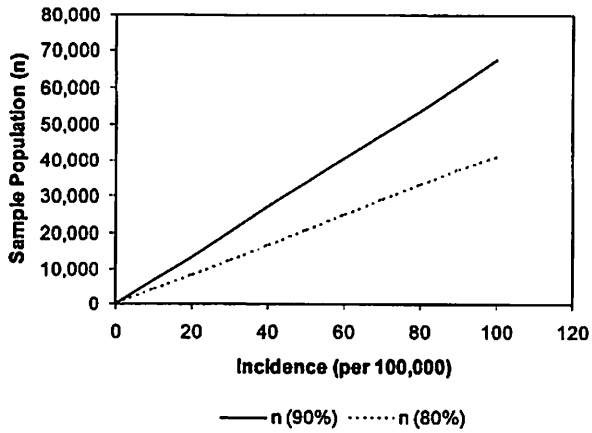


Figure 2. Sample size estimation by binomial approximation. This plot is derived from the sample size formula presented in Equation 3. The assumed tolerance level B is 0.0002 and the assumed confidence levels within the legends are expressed in percentage.

Following the establishment of the screening model, the main experimental parameter to be addressed in the population analysis became whether the Interstron/Biodecan product can effectively assist in the identification of high risk patients affected by breast cancer. According to the screening algorithm defined in the clinical protocol, medical attention was devoted primarily to the At Risk group with Interstron activity >1200 IU_{JPA}. A measure of the effectiveness of the screening is reflected by the proximity of the measured incident rate to that of the general incident rate of breast cancer in Mexico. In this respect, a binomial approximation was used to estimate the sample size for the population analysis (Equation 3), where p stands for annual population incidence of breast cancer, and B presents the tolerance of analysis. Equation 3: $n= 2pq\ (Z_{\alpha/2}/B)^2$

Based on figures obtained from Globoscan® and WHO mortality databases, we estimate the annual incident rate of breast cancer in Mexico to be 30-60 per 100,000 (32,33). As shown in Fig. 2, at a tolerance of 0.0002, such incident rate requires a sample size between 12,000 to 40,000 subjects in order to ensure 80 to 90% confidence level. In this report, distribution values from approximately 33,000 women are shown.

Inclusion/exclusion criteria. Since Mexican women are known to have early onset of breast cancer (7,8), the age group of interest was set at 35 to 70, including younger women than the typical target group for mammography. For the clinical study, healthy and Mexican women with cancer in this age group were selected at random, with the exclusion of pregnant women, nursing mothers during the first 4 months of lactation, and patients undergoing chemotherapy, radiation treatment or taking cytotoxic agents, anti-estrogens or selective estrogen receptor modulators. For the population surveillance program, all women of the same age group were randomly enrolled based on voluntary basis. The only exclusion criterion was women with serious contagious diseases.

Case/control criteria. During the initial patient enrollment of the clinical study, case control was assigned based on the available medical diagnosis of the patient which comprised of medical examination, mammography, ultrasound, and biopsy analyses. A case was defined to be a woman with confirmed or suspected malignant breast cancer and control was defined to be randomly recruited women under the same age group with no confirmed breast cancer. Later, the case/control criteria were re-evaluated after full medical examination and analysis of all patients by the Mexican General Hospital. Based on the biopsy reports, 18 out of the 32 women in the case group were confirmed with malignant stage I to IV breast cancer whereby these women were considered true cases. Among the 31 women in the control groups, 15 had no breast cancer related symptoms, while 17 had one or more symptoms such as pain in the breast or nipple secretion. Overall, this information was taken into consideration in the statistical analysis presented in the Results and Discussion sections.

Masking. Patient information was blinded to the investigators who conducted the Interstron activity analysis. Case reports were made available only after all activity readings were determined.

Ethical considerations. Ethical Guidelines (WMA, 2000) from The World Medical Association in Medical Research involving human subjects were followed throughout the entire study (34). Members of ICT reviewed the design of the clinical trial and approved the protocol. An interview was conducted prior to formal enrollment of participating women to determine baseline patient information and to extend an invitation to enter the study on a strictly voluntary basis without affecting the ongoing diagnosis and treatment of each participant. Information collected from each patient included physical condition, examination of mammary glands, reproductive history, clinical history, family history, life style and dietary habits. All collected information was entered into a coded database, strictly maintaining the identity and confidentiality of each participant. Prior to patient recruitment, clinical personnel involved in the study were trained in Good Clinical Practices, including the adequate completion of all related and proper documentation relevant to the study.

Coordination and monitoring. Recruitment for the clinical cohort was coordinated by the Mammography Clinic of the Oncology Service in collaboration with the General Hospital of Mexico in early 2002. The overall quality control and monitoring of the clinical trial were conducted by the Investigation Science and Technology (ICT, S.A. de C.V.) in Mexico. The population analysis program was initiated in April 2003 under the supervision of the Secretary of Health of Mexico. As of July 18, 2003, the program had completed Interstron/Biodecan analyses on 32,958 Mexican women.

Medical profiling of patients enrolled in the clinical study was performed at the General Hospital of Mexico. Mammographic images were obtained by using a Cenovision Mod 2230491 General Electric equipment. Mammograms were interpreted by the Imaging Service in coordination with the Oncology Service at the General Hospital. Mammary tumor biopsies were practiced on selective tissue samples

using techniques such as the fine needle aspiration technique (BAAF). The analyses and interpretations of the tissue samples were performed at the Pathology Department of the General Hospital of Mexico.

Execution of analysis program. The Interstron/Biodecan test includes two main sub components: one for blood sample collection and the other for an immuno-capturing test to determine the Interstron activity levels. Due to the highly sensitive nature of the test, samples were only analyzed in designated test centers with fully trained staff. During the clinical study, blood samples were collected in a glass vacuum tube containing anticoagulant and sent to a testing facility for analysis by air courier. In the on-going field test in Mexico, all blood samples were collected with a blood collection kit included in Interstron/Biodecan set and sent to a standardized designated test center for the estimation of Interstron activities. Each sample is identified by a unique bar-code, which was recorded by clinicians at the time of blood withdrawal as the reference sample ID. At the test centers, blood samples were examined for damage and quality of blood preservation prior to processing. Samples with visible disintegration or damage were noted and rejected for further analyses.

Monoclonal antibody production. Large scale monoclonal antibody production of Interstron I (anti-LAPase, EC3.4.11.1) and Interstron II (anti-NDPK, EC 2.7.4.6) was carried out using two Biovest (formerly Unisyn Technologies, Inc., Tustin, CA) hollow-fiber bioreactors Cell-Pharm System 1500™, according to standard procedures (35-39). Briefly, human/mouse hybridomas cells were first expanded in tissue culture flasks (Corning, Corning, NY) at a seeding cell density of $5x10^4$ cells/ml using a growth medium composed of Gibco™ RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT), penicillin-streptomycin (10^3 U/ml - 10^3 µg/ml), sodium pyruvate (1 mM) and non essential amino-acids (0.1 mM). Cells were inoculated into the Bioreactor after reaching a density of $1x10^9$ viable cells/ml. Cell growth within the hollow fiber matrices was monitored through metabolic parameters such as glucose utilization rate (GUR) (35,36,38). The extra capillary harvest was collected and concentrated using Pellicon Ultrafiltration Cassette System (Millipore, Billerica, MA). Finally, after ultrafiltration, antibodies in the resulting retentate were purified by using a Protein G (Amersham Biosciences, Piscataway, NJ) column and then dialyzed against PBS.

Activity determination. The principle behind the Interstron/Biodecan blood test resides in simultaneous capturing of two cellular factors namely NDP-Kinase (NDPK) and LAPase, which have increased biological activities when stimulated by circulating levels of 17ß-Estradiol (estrogen) (24). Interstron I (EC3.4.11.1) (28) and Interstron II (EC 2.7.4.6) (27) are two monoclonal antibodies against LAPase and NDP-Kinase respectively (29,30), which in tandem capture two enzyme complexes with a specificity of 89 to 99% relative to other cytosolic proteins (95% CI, results not shown), the activity of this complex is subsequently estimated with the aid of a specific substrate and a Universal Reference

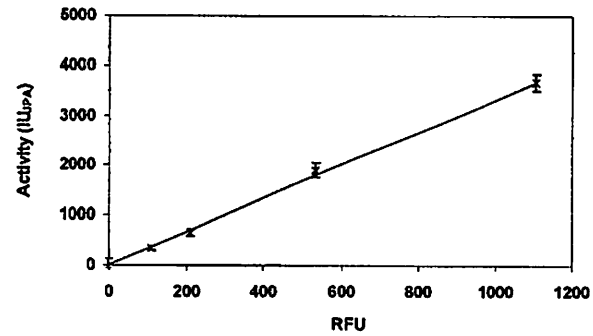


Figure 3. Analytical sensitivity of Interstron/Biodecan method. The results presented are derived from the testing of reference antigenic standards on 3 plates with 8 curves per plate and duplicated measurements for each curve. RFU stands for Relative Fluorescence Intensity. The sensitivity is calculated as the ratio of standard deviation to average. The CV% value was computed as the ratio of standard deviation at each point with respect to the overall reference range.

Antigenic Standard provided in the Interstron/Biodecan set. One JPA unit of Interstron complex (IU_{JPA}) converts the Interstron/Biodecan reaction substrate at a rate of 0.2 nmole per min at a pH range of 7.0 to 7.4.

At designated test centers, Interstron activity was determined by staff trained to perform Interstron/Biodecan analyses. In summary, a 6-8 ml blood sample was collected from each individual in a glass Vacutainer tube containing 1.5 ml of an anticoagulant solution composed of 22.0 g/l sodium citrate, 8.0 g/l citric acid, and 24.5 g/l Dextrose. Fresh blood samples were delivered to designated test centers by air courier within 7 days. Upon arriving, the blood samples were first spun at 450 x g (~15000 rpm) at 4°C for 20 min. Then, the separated plasma is centrifuged once again at 4900 x g (~5000 rpm) at 4°C for 10 min to remove any remaining cell debris. After the second centrifugation, a quantifiable immuno-capturing enzymatic assay was performed on the plasma samples according to the instruction information supplied by Interstron/Biodecan products. The plate readings together with plate arrangements and sample bar-code information were delivered to a universal database (a component of Nova Integratum system) which computes the activity levels (expressed in IU_{JPA}) with a systematic calibration according to manufacturing lot numbers. Standard curves were also monitored in terms of linearity and consistency as compared to the inter- and intra-plate variations determined prior to the release of the products. Fig. 3 demonstrates the observed variations from a typical quality control test on the reference standard. Assay sensitivity displayed here varies from 1.3 to 4.5% relative to the reference range, and varies from 4.5 to 14% (near lower detection limit) relative to the average activity at each reference point.

Computational analyses. Samples collected for the clinical studies were analyzed in quadruplets. The sample activity level is expressed as the average plate reading translated into IU_{JPA} according to the Universal Reference Antigenic Standards. Field test samples were performed in duplicates with stringent Quality Assurance (QA) criteria on both reference and reading quality. If two duplicated readings for

Table I. Descriptive characteristics of the patient cohort.[a]

Characteristics		Tumor (-)	Tumor (+)
Age	35-49	21 (66%)	19 (61%)
	50-70	11 (34%)	12 (39%)
Onset of menarche	<13	15 (47%)	15 (48%)
	>13	17 (53%)	16 (52%)
Age at first birth	<23	19 (59%)	14 (45%)
	>23	12 (38%)	13 (42%)
	NA	1 (3%)	4 (13%)
Breast feeding	Y	28 (88%)	22 (71%)
	N	3 (9%)	5 (16%)
	NA	1 (3%)	4 (13%)
Parity	>3	22 (69%)	17 (55%)
	<3	9 (28%)	10 (32%)
	0	1 (3%)	4 (13%)
HR therapy	Y	2 (6%)	2 (6%)
	N	30 (94%)	29 (94%)
Breast cancer in relatives	Y	5 (16%)	5 (16%)
	N	27 (84%)	26 (84%)
Red meat	Y	26 (81%)	22 (71%)
	N	6 (19%)	9 (29%)
BMI	<30	25 (78%)	21 (68%)
	>30	5 (16%)	8 (26%)
	NA	2 (6%)	2 (6%)
Smoking	Y	7 (22%)	4 (13%)
	N	25 (78%)	27 (87%)
Alcohol intake	Y	2 (6%)	3 (10%)
	N	30 (94%)	28 (90%)
Exercise	Y	6 (19%)	4 (13%)
	N	26 (81%)	27 (87%)

[a]Patients were grouped according to presence or absence of tumors. The classification of the two groups was based on clinical reports which included prognoses ranging from clinical examination, mammography, ultrasound and biopsy. The tumor present group includes patients with stage I to IV breast cancer, benign tumors or tumors of undetermined malignancy. HR, Hormone replacement therapy; BMI, body mass index calculated as kg/m^2. Review of breast cancer in family members included mother, grandmother, daughters, sisters, aunts and cousins. Y, yes; N, no; NA, not applicable.

a single sample failed on reading consistency, the sample was re-tested. If the reference on a particular plate displayed poor linearity or out of regular QA range, the whole plate was re-tested.

Baseline patient information pertaining to the clinical study was kept abreast from investigators at the testing facilities until after analysis data were delivered and the subsequent cross-matching of data by recruiting centers was completed. Clinical thresholds were thereafter determined based on the available pathophysiological reports and the cohort was classified into Risk groups according to Interstron activity levels. Taking into account the available pathological profiles of the participants in the clinical study, the statistical significance of sample activities displayed in different groups was determined with a series of t-tests. Clinical profiles of participants to the ongoing mega field program have not yet been disclosed, therefore, no statistical analysis of the testing population is currently available, other than the activity distribution.

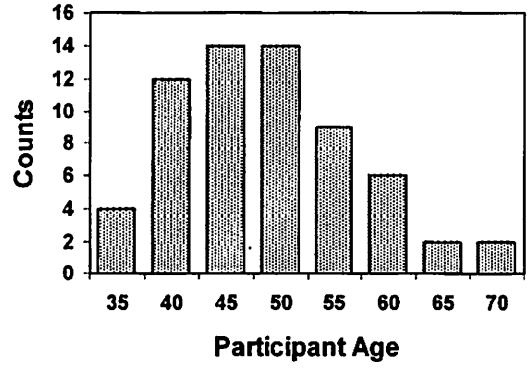


Figure 4. Age distribution of cohort. Age distribution of cohort demonstrates an approximate Gaussian distribution with lower frequencies of participants between 35 to 40 and 65 to 70 years of age, respectively.

Results

Characteristics of patient cohort for clinical study. Several well-known aetiological factors have been ascribed to breast cancer, including early onset of endogenous estrogen intake, menarche, low parity and lack of breast feeding, older age at first child birth, family history of breast cancer, unhealthy dietary habits, high BMI and aging (40-42). As shown in Table I, the clinical cohorts encompass a balanced representation of all the risk factors aforementioned. Based on the pathological report, the cohort is subdivided into tumor (+) and tumor (-) groups. The tumor (+) group includes patients with either benign or malignant tumors and also includes three patients with tumors of undetermined types. The tumor (-) group includes all participants with normal pathology. One of the most recognized risk factors for breast cancer is aging (40,42). The overall age distribution of the cohort is approximately normal, with median centered around 45 to 50 years of age (Fig. 4). The proportion of the tumor (+) versus tumor (-) candidates was kept roughly the same in the ≤50 age groups to avoid possible bias that may be introduced by larger proportion of aged participants in one group.

The cohort selection also has a normal distribution in terms of age at menarche. The average onset of menarche was 13.0±1.7 years, which is similar to the reported median age of 12.43 for US girls (44). About an equal proportion of tumor (+) versus tumor (-) participants were selected in the <13 and ≥13 age groups at menarche. Average age at first birth was 22.3±5.4 for the cohort, which is close to the reported average age of Mexican women at first child birth.

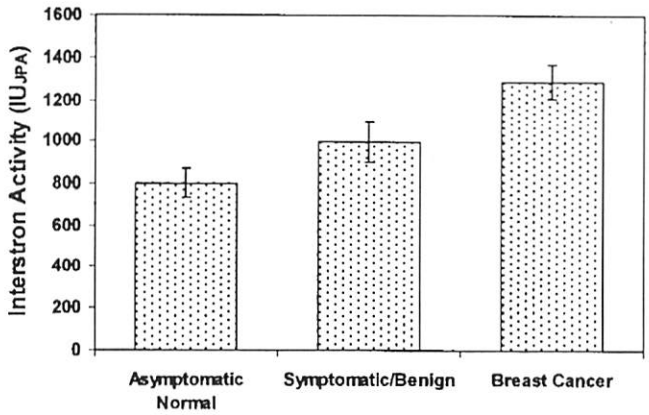
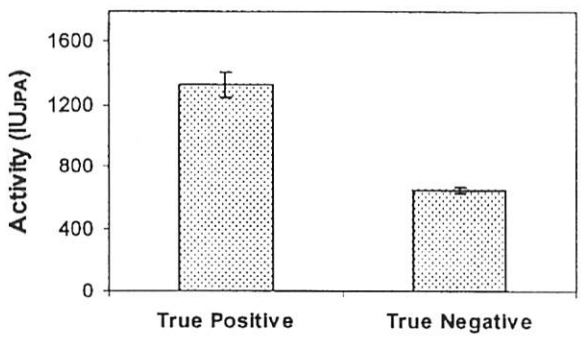


Figure 5. Segregation pattern based on disease progression. The participants were divided into asymptomatic normal, symptomatic or benign, and breast cancer groups based on the biopsy results and medical examination of the patients by the General Hospital of Mexico. Displayed are the averaged Interstron activities with error bars indicating the standard error for each group.



Biopsy Results

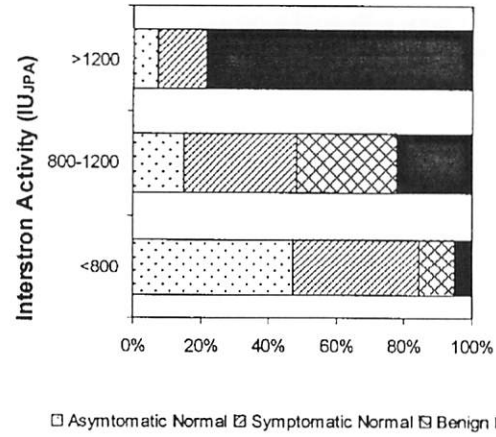
Biodecan®

A: True Positive • Breast cancer stage I-IV • Activity > 800 IU_{JPA}	**B: False Positive** • Asymptomatic • Activity > 800 IU_{JPA}
C: False Negative • Breast cancer stage I-IV • Activity < 800 IU_{JPA}	**D: True Negative** • Absence of breast cancer • Activity < 800 IU_{JPA}

Figure 6. Sensitivity/specificity and establishment of thresholds. Presented is the categorization scheme used to compute sensitivity and specificity for the clinical trial. Sensitivity of the method is 0.94, which was determined as the ratio of group A over the summation of groups A and C. Specificity of the method is 0.83, which was determined from the ratio of group D over the summation of groups D and B. The dual thresholds are set at 800 and 1200 IU_{JPA} respectively based on apparent values of the true positives and true negatives. Plotted are the average Interstron activity values from true positive and true negative groups, with error bars indicating the standard error. By two-tailed heteroscedastic t-test, the separation between the true positives and true negatives is 521.8 IU_{JPA} at α=0.05.

Among the 63 participants, 5 women never had children. Among the 58 women who had offspring, more than 85% breast fed their children, supporting the fact that majority of Mexican women practice breast feeding.

Establishment of thresholds. Since Interstron activity is a continuous variable, it would be difficult to conform it to a conventional prediction algorithm that has a single threshold



Risk Group	Criteria	Propensity
At Risk Group	Activity > 1200 IU_{JPA} & Abnormal symptoms	Malignant
Grey Risk Area	800 IU_{JPA} < Activity < 1200 IU_{JPA} & Abnormal symptoms	Tumor
Normal Otherwise Healthy	Activity < 800 IU_{JPA}	Normal

Figure 7. Screening criteria and composition of classified groups. As a result of the two thresholds set at 800 and 1200 IU_{JPA}, three groups were classified: (i) Healthy Otherwise Normal, (ii) Grey Risk group, and (iii) At Risk group. The transient nature of the Grey Risk group is reflected in the heterogeneity of patient composition.

determining binary outcomes. When the clinical cohort is divided into three groups: (i) asymptomatic normal, (ii) symptomatic normal or benign and (iii) cancer based on the health state of the subject, it is apparent that Interstron activity is a parameter associated to the progression of breast cancer (Fig. 5). Briefly, patients with well-defined malignancy have an average Interstron activity above 1200 IU_{JPA}, while the normal asymptomatic participants had typical Interstron activities around 800 IU_{JPA}. Therefore, 800 and 1200 IU_{JPA} appear to be the relative tangible choices for threshold values.

Clinical sensitivity and specificity. Fig. 6 presents evaluation of sensitivity and specificity of Interstron/Biodecan assay. Clinical trial participants were grouped into true positive, true negative, false positive and false negative groups based on pathological findings and Interstron activity levels. To establish consistent disease staging, biopsy results were used as the referral standard to define the malignancy in the patient population. Interstron activity was artificially parted at 800 IU_{JPA} to conform to the binary prediction scheme. Therefore, the true positive group includes participants with well-defined stage I to IV breast cancer and Interstron activity >800 IU_{JPA}. Accordingly, the false positives are participants with no cancer symptoms, normal mammary glands and Interstron readings >800 IU_{JPA}. Overall, after the cohort was duly categorized and evaluated, the sensitivity of the method was determined to be 0.94, while specificity was calculated to be 0.83.

The separation between the true positive and true negative is well above 400 IU_{JPA} (p=0.002, two-tailed heteroscedastic

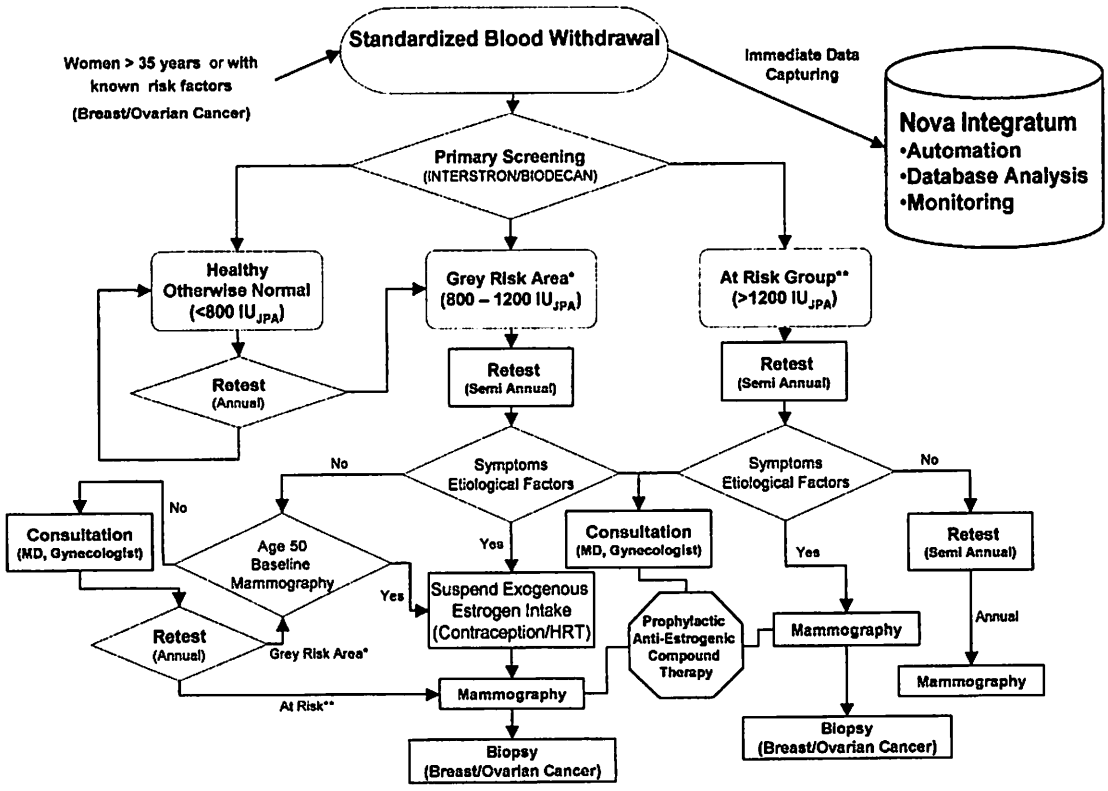


Figure 8. Proposed clinical surveillance algorithm. The proposed breast cancer screening algorithm using Interstron/Biodecan method has been integrated into the design of both semi-automated and fully-automated sample analysis of whole blood samples.

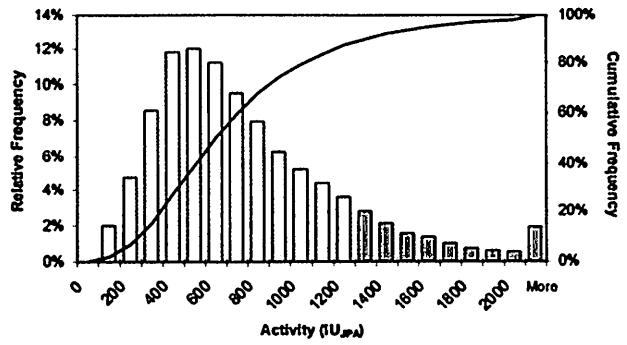


Figure 9. Activity distribution in Mexican women from current analyses. The presented data were collected between April 2 and July 18, 2003. Included are 32,958 entries verified for sample reading consistency and reference quality. Population mean of Interstron activity is 708.4 IU$_{JPA}$, with 67.8% of participants having Interstron activity <800 IU$_{JPA}$ and 12.7% of participants having Interstron activities >1200 IU$_{JPA}$.

t-test), confirming the adequacy of 1200 IU$_{JPA}$ as the second threshold (Fig. 6). Screening criteria were subsequently developed based on the dual thresholds model and women were classified into 3 groups based on their Interstron activity levels and presence of symptoms: (i) Healthy Otherwise Normal, (ii) Grey Risk Area, and (iii) At Risk group. Details of risk group classification and composition of the risk groups are depicted in Fig. 7.

Establishment of population surveillance algorithm. Following the classification of risk groups determined from the clinical study, a surveillance algorithm was put forward in seeking early detection of breast cancer with the assistance of Interstron/Biodecan a quantitative immuno-capturing assay (Fig. 8). A surveillance algorithm similar to Fig. 8 has now been pursued in a field practice in Mexico. During the 3-month period between April and July 18th, 2003, Interstron activities had been determined in ~33,000 Mexican women. Since image based analysis and other pathological examinations can not be completed in the same time frame, not all clinical data are yet available. However, the trend of risk group segregation in the 33,000 women can be seen from the distribution of Interstron activities in Fig. 9.

Discussion

Risk groups and breast cancer dynamics. Cross-matching of the reported pathology classification with that of the plasma Interstron activity levels led to the definition of 3 distinct groups among all patients recruited in the clinical study. Definitions and classifications of the 3 risk groups are illustrated in Fig. 7. The design rationale behind such a 3-tiered algorithm is to allow the relatively homogenous grouping of the normal versus cancerous population in low and at risk levels, while properly reflecting the transitional population between the two phases characteristic of the heterogeneous Grey Risk group.

According to the proposed classification as shown in Fig. 7, the Healthy Otherwise Normal Group (<800 IU$_{JPA}$) as determined by the quantitative estimation of Interstron activity levels was consistent with the pathology classification of healthy women, with the exception of one case. The referred exception represented the sole false negative

case, ascribed as a malignant carcinoma in stage III-IV with Interstron activity of <800 units. The exact cause of the false negative value could not be identified without knowledge of the particlar entire treatment history. However, from our previous investigations, we have found evidence that certain cancer treatment such as radiation therapy can suppress the Interstron activity (unpublished data). This observation is consistent with the recent findings from Brookhaven National Laboratory on plasma proteinase MMP-9, which is usually expressed in elevated levels in cancer patients (47). This group has reported that plasma proteinase MMP-9 expression is suppressed in breast cancer/lung cancer patients during the first 2 weeks of radiotherapy (48). Nonetheless, it is conceivable that other factors such as chemotherapy or medication may also exert adverse effects on Interstron activity.

Women within the Grey Risk Group constitute a heterogeneous population of normal, benign and malignant cases with complex activity varying between 800 and 1200 IU_{JPA} (Fig. 7). Interestingly, this group possesses all the characteristics of a true transitional population with a differential pathophysiological and clinical distribution (49). Based on Interstron activity values, women within this group may represent the potential conversion of both asymptomatic women with yet undetected maladies as well as of symptomatic benign lesions to potentially malignant breast carcinomas.

The Grey Risk group can be further streamlined into a Tumor Risk group with the assistance of clinical information. Taking into account the presence of both abnormal mammary glands and presence of symptoms clinically reported during enrollment, this population can be depurated revealing a predominant tumor-bearing population. About half of these tumors are of benign pathology and the other half were identified as malignant. More interestingly, most of the malignant cases are of early stage (I-II) carcinomas, reaffirming the transitional nature of the Grey Risk group.

Similar to the Healthy Otherwise Normal group, the At Risk group (>1200_{JPA}) is relatively homogeneous in its composition (Fig. 7). Women within this group had clear presence of symptoms, abnormal mammary glands and poor histopathological outcome of tumour biopsies (malignant tumours in Stage I-II or III-IV respectively). There were 3 individuals in the At Risk group that were classified as pathologically normal. Two out of these three cases had either presence of breast cancer symptoms or abnormalities in the mammary glands, the nature of which was to be determined by follow-up examinations. Therefore, only one patient was considered a true false positive. Compared to the tumor risk group streamlined from the Grey Risk Area, women in the At Risk group appeared to have higher Interstron activity readings and were in advanced stages of breast cancer.

Quantitative interpretation of risk factors. The available participant reports on the clinical cohort enable us to gain some quantitative insights into the common risk factors for breast cancer (Table II). Overall, the average activity for the asymptomatic normal group is 800±65 IU_{JPA} with the breast cancer patient population averaging 1289±81 IU_{JPA}. A similar difference is observed when the cohort is subdivided into age groups of >50 years and <50 years. Consistent with the current age dynamic trend of breast cancer incidence the older group (>50) has higher Interstron activities than the younger group, correlating with the more incidence of advanced breast cancer in this group (40-43,50). It is noteworthy that the average of the asymptomatic normal group is about the same for both the older and younger populations, indicating that the baseline Interstron activity is age-independent.

Table II. Interstron activity levels in different participant groups.

Characteristics		Avg. activity (IU_{JPA})	Δ_0	p-value ($T \leq t$)	DF
Age					
≥50		1156.1	100	0.43	54
<50		967.2			
<50	Cancer	1091.7	100	0.09	14
	AN	809.8			
≥50	Cancer	1486.6	400	0.05	13
	AN	785.9			
Age at first birth					
≥30		1162.3	100	0.34	6
<18		851.4			
BMI					
≥30		1169.7	100	0.36	20
<25		931.3			
Tumor type					
Malignant		1289.1	100	0.08	26
Benign		993.9			
Health state					
Breast cancer		1289.1	200	0.009	25
AN		800.0			

AN, Asymptomatic normal; BMI, body mass index calculated as kg/m^2. The p-values cited are the results from two-tailed heteroscedastic t-test examining the significance of the hypothesized mean difference (Δ_0), and DF represents the degree of freedom.

Due to the limited availability of clinical reports, it is difficult to make conclusive correlations between Interstron activities and detailed population characteristics at this stage. However, we did observe a higher average activity in patients with BMI >30 (1169.7 IU_{JPA}) versus patients with BMI <25 (931.3 IU_{JPA}), consistent with other literature findings indicating that breast cancer is associated with obesity (45,46). The age of women at first child birth also seems to place a risk factor in breast cancer development with the older first time mothers (1162.3 IU_{JPA}) having higher Interstron activities than the younger mothers (851.4 IU_{JPA}). Overall these observations are consistent with past and present risk factors associated to the early pathophysiological events leading to breast cancer (50-52).

Integration of population surveillance algorithm and advance in methodology. Following the delineation of the Interstron activity thresholds and classification of risk groups, a systematic screening algorithm has been proposed (Fig. 8). During initial testing, women are grouped into the Healthy Otherwise Normal, Grey Risk Area, and At Risk categories pending on their Interstron activity levels. It is suggested that the Healthy Otherwise Normal group be examined every year. If the test result reveals an intermediate level of activity, women should be recalled for a physical examination and confirmatory test 6 months after the initial testing. If the confirmatory test assesses the woman in the same risk category, she will be assigned to a physician to closely monitor Interstron activity every 6 months. At the same time, women can be given professional advice on life style and dietary habits in order to reduce potential cancer risk. Priority is given to the At Risk group (>1200 IU_{JPA}), which has the highest potential to develop malignant tumors. It is advised that these women undergo mammography, ultrasound or other techniques to detect and verify the presence of potential tumor growth. After prognosis, the Interstron/Biodecan test could still play an active role as to evaluate treatment efficacy and predict potential relapses by monitoring overall trend changes in the three main group distribution described according to their corresponding putative thresholds.

A similar algorithm has been adopted in the population analysis in Mexico involving more than 33,000 women. Preliminary population analysis reveals that ~12.7% of women are within the At Risk group (Fig. 9). Currently, these women are given prioritized medical attention in terms of follow-up examinations and mammography, whereby Interstron/Biodecan may function as a quantitative surveillance assisting tool towards an early detection of breast cancer. In this regard, taking into account the actual analytical rate and logistics of the Interstron/Biodecan assay, equating to 10,000 participants screened per month at a single testing centre, the target population for a more comprehensive evaluation was considerably reduced to 1 in 8 women. This proportion singles out the effectiveness of Interstron/Biodecan as a viable quantitative screening tool to assist mammography and other image technologies to selectively focus resources within the At Risk Population group.

In light of the current demand for a greater efficiency in populational breast cancer screening, an automated multi-tasking Interstron Processing Analytical system Nova Integratum AMT/3000 is in development. The robotic system is designed to further reduce labor intensity and increase the level of systematic standardization though centralized data handling. In the near future, more field testing might unfold the presence of yet additional risk factors as to provide additional quantifiable correlations between overall exposure to both endogenous and exogenous estrogen (reflected as an estrogenic window during a woman's lifespan) and Interstron activity levels.

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