Journal of Cancer Science and Clinical Oncology
Volume 5 | Issue 2
ISSN: 2394-6520

Research Article | Open Access

Profile of Tumor-Associated Cytokines among Breast Cancer Patients: A Preliminary Study

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Citation: Streckfus CF, Nwizu N, Streckfus CG, Arreola D (2018) Profile of Tumor-Associated Cytokines among Breast Cancer Patients: A Preliminary Study. J Cancer Sci Clin Oncol 5(2): 206

Abstract

Objective: The link between disease and the oral cavity represent clinically important factors that have given rise to the interest in using saliva as a diagnostic fluid for systemic diseases. The objective of this study was to detect and quantify cancer related cytokines in the saliva of women with breast cancer and compare their concentrations to a healthy cohort.

Methods: A multiplex analysis of eight different cytokines (IL-1ß, IL-6, IL-8 IL-10, IFN-γ, EGF, MCP-1 and VEGF) was performed on stimulated whole saliva specimens from cancer patients (n = 36) and from healthy women (n = 20). All specimens were assayed in triplicate on the Bioplex multi-analyte suspension array. Total protein quantification of the specimens was performed using the BCA technique. Statistical analyses for descriptive, graphical and mean value comparisons were performed using the SPSS program.

Results: The assay proved the presence of all the cytokines in both sets of specimens. In comparison to the healthy cohort, saliva IL-6, IL-10, EGF, MCP-1 and MCP-1 concentrations were elevated while IL-1ß, IL-8 and IFN-γ were lower among the breast cancer subjects. VEGF remained unchanged. We also noted that these cytokine concentrations could be modulated as many of them exhibited a decrease in concentration after the tumor was removed (one-year post-op). Conclusion: This study indicates that the presence of cancer related cytokines in saliva might have utility for monitoring patient response to chemotherapy.

Keywords: breast cancer; cytokines; growth factor; tumor microenvironment; saliva

List of abbreviations: IL-1ß: Interleukin one beta; IL-6: Interleukin six; IL-8: Interleukin eight; IL-10: Interleukin ten; IFN-γ: Interferon-gamma; EGF: Epidermal Growth Factor; MCP-1: Monocyte Chemoattractant Protein - one; VEGF: Vascular Endothelial Growth Factor; SWS: Stimulated whole Saliva; TP: Total Protein; Std. Dev.: Standard Deviation; Std. Err.: Standard Error of the mean; C.I: Confidence Interval; Min.: Minimum; Max.: Maximum; U: Mann-Whitney U; CMF: cyclophosphamide; methotrexate and fluorouracil; AC: Adriamycin and Cyclophosphamide.

Introduction

Proteins indicative of disease have been observed in saliva for many years [1,2]. For example, the presence of breast cancer related proteins in saliva and mRNA have been described in the work of Streckfus and Wong [1-3]. One such group of cancer related proteins, the signaling proteins, is pertinent to immune response, cancer development, angiogenesis and they are present in the serum and saliva via microvesicles, exosomes and solubilized particulates (e.g., hormones, cytokines, chemokines and growth factors).

The immune response to a neoplasm is very intense and involves numerous signal molecules. Simply stated, tumor cells secrete factors that alter stromal cells, which in turn secrete signaling proteins and/or cytokines that affect the tumor cells [4]. In addition, tumor-associated macrophages also play a major role in cytokine production and tumor progression [5].

Macrophages invade tumors and once embedded secrete cytokines that attract a barrage of cells to the tumor site. Macrophages are modified to promote tumor growth, inflammation, angiogenesis and metastasis through cross talk with other migrating and resident stromal cells such as fibroblasts and adipocytes. The inflammatory signal cascade can exacerbate tumor growth and contribute to a tumor's genetic heterogeneity [4-9]. Consequently, the cancer microenvironment is a rich source of target of cytokines, chemokines and growth factors [9-12].

These wide-traveling cytokine molecules are then available for detection in the biological fluids, including saliva. Therefore, it

is not unlikely that disease related signal proteins like cytokines are non-specifically trafficked through the salivary glands and then secreted in saliva. The shared epithelial nature of breast carcinoma and the salivary glands may be the link between systemic disease markers and their presence in the saliva. Consequently, certain cytokines will be present at higher or lower concentrations than normal levels if the patient suffers from a systemic disease such as breast cancer [1].

Taken together, the purpose of this investigation was to determine if salivary cytokines, chemokines and growth factors are altered in concentration secondary to carcinoma of the breast. The main objectives of the present study are to: (i) identify and compare the abundance of cytokines, chemokines and growth factors present in the saliva of breast cancer and a normal control population and identify any possible correlations between cytokines present in saliva among healthy and diseased populations and (ii) characterize the cytokine profiles of various tumor subtypes [4-12]. Henceforth, in order to save space and decrease redundancy, cytokines, chemokines and growth factors will be collectively referred to as either cytokines or analytes.

Materials and Methods

Subjects

The study consisted of two groups of women all of which signed the Institutional Review Board approved consent form HSC-DB-05-0394. Group I was a control group. This group consisted of healthy, asymptomatic individuals with no previous history of cancer. Health status for the control group was determined by questionnaire. The questionnaire included information concerning their age, race, tobacco usage, pharmacological, medical and gynecological histories. Saliva was collected at the time of the visit.

Group II, the breast cancer cohort, had saliva specimens collected at their first patient visit prior to any treatment and similar to the control group, they were administered a questionnaire. Their cancer status was determined by their respective pathology report; whereby, staging and nodal status were assessed according to the criteria set forth by the American Joint Committee on Cancer [13,14]. Hormonal and Her2/neu receptor status were also provided by the pathology report.

Seven subjects from the cancer cohort had saliva specimens collected one year after diagnosis and after their respective chemotherapeutic regimens were completed. Their specimens were assayed to determine if the analyte concentrations were modulated after therapy.

Specimen collection

Stimulated whole saliva specimens were collected for a five-minute period using a cube of gum base as a stimulant following standardized collection procedures [11]. Upon collection, the specimens were aliquoted and frozen for analysis. Salivary flow rates were determined gravimetrically. All specimens were collected in the morning thereby controlling for any possible effects that circadian rhythm may produce in marker concentration.

Total protein assay

The frozen specimens were thawed and the saliva specimens were thawed, centrifuged and analyzed for total protein and the analyte concentrations. Samples of saliva were assayed for protein using the bicinchoninic acid method (Pierce Chemical, Co.) which is a highly sensitive and selective detection reagent for the cuprous ion. This method measures protein concentrations from 0.5-20 mg/ml. In this assay, bicinchoninic acid serves as a chelating agent for Cu+1 forming a color complex in the presence of protein. Aliquots of saliva (100 µL) were placed in microtiter plates and the Pierce BCS protein assay reagent added to the wells. Samples were incubated for 30 minutes at 37 0C and the optical density read at 562 nm in a microplate spectrophotometer. The final concentration of each substance was derived from a standard curve and data was expressed as mg/mL.

Cytokine analysis

In this study, a panel for cytokines was used to assay the saliva of patients, ranging from healthy controls to stage IIb breast cancer. The targeted cytokines were chosen based on cytokine involvement in breast cancer as reported in the literature [6-9]. The panel targets IL 1-β, IL 6, IL 8, IL 10, IFN-γ, EGF, MCP-1 and VEGF.

Stimulated whole saliva was cleared by centrifugation at 14,000 g for 10 minutes at 4°C. The clear supernatant containing the soluble proteins was then processed as suggested by the manufacturer for the cytokine multiplex assay (LincoPlex®, St. Charles MO) [15]. The assay was read on the Bio-Plex® 200 suspension array system (BioRad, Hercules CA) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the SPSS™ statistical software package. The data were analyzed from four different perspectives. Initially, the saliva analyte concentrations were summarized for each analyte and descriptive analyses were conducted for the demographic and supplemental data obtained from the questionnaire. The focus was on race, medical status, tobacco use, medication usage, and menopausal status with respect to analyte concentrations. The data among the cancer cohort were

summarized by tumor type, staging, and nodal status. Due to the small number of women in the cancer group, the number of sub-categories for primary tumor size (T) and nodal status (N) were collapsed. The primary tumor categories were dichotomized to less and greater than 2.0 cm, while nodal status was reduced to node negative and node positive, respectively.

Non-parametric statistics were employed throughout the study due to large differences in variances, lack of normality in distribution and outliers. More importantly, the data was better represented by the median as compared to mean differences. Consequently, the Mann-Whitney "U" and the Kruskal-Wallis test were used for pairwise and multi-group assessments respectively. The alpha level was 0.05; however, alpha values ranging from 0.06 to 0.09 were also reported.[16]

Possible associations among the salivary cytokine levels as well were assessed by Spearman's Rho correlation coefficient.

Results

The independent variable results for the social demographics, anthropometric, gynecological history, comorbidity and medication usage are provided in Table 1. Briefly, the respectively. The alpha level was 0.05; however, alpha values ranging from 0.06 to 0.09 were also reported [16]. Possible associations among the salivary cytokine levels as well were assessed by Spearman's Rho correlation coefficient.

Characteristic	Control	Malignancies	Significance
n (56)	20	36	--
Age	42 (±10)	53 (±10)	n.s.
BMI	27 (±5.3)	31 (±8.2)	n.s.
Race			n.s.
Caucasian	8	13	--
African American	12	23	--
Tobacco Usage			n.s.
Yes	6	13	--
No	14	23	--
Alcohol Usage			n.s.
Yes	11	12	--
No	9	16	--
Menopausal Status			**$X^2 = 5.83, p<0.02$**
Premenopausal	13	11	--
Postmenopausal	7	24	--
Birth Control Usage			n.s.
Yes	2	3	--
No	18	29	--
HRT			n.s.
Yes	6	6	--
No	14	23	--
Hysterectomy			n.s.
Yes	2	9	--
No	18	23	--
First Menses	13 (±2.1)	13 (±1.8)	n.s.
Number Pregnancies	2.1 (±1.1)	4 (±2.8)	**U = 457, p<0.004**
Live Births	2 (±1.3)	4 (±2.7)	**U = 497, p<0.001**
Age First Birth	22 (±5.2)	20 (±4)	n.s.
Systemic Diseases			**$X^2 = 4.19$ p<0.04**
Yes	4	16	--
No	15	16	--
Medications			**$X^2 = 4.56, p<0.03$**
Yes	6	20	--
No	14	12	--

Abbreviations: BMI = Body Mass Index; HRT = Hormone Replacement Therapy; n.s. = Not Significant; X2 = Chi Square; U = Mann Whitney U.
Table 1: Descriptive statistics for social demographics, health status, gynecological and health history and medication usage

The independent variable results for the social demographics, anthropometric, gynecological history, comorbidity status and medication usage are provided in Table 1. Briefly, the healthy cohort (n=20) exhibited a mean age of 42 (±10) years and was composed of twelve African-American and eight Caucasian women. The average body mass index (BMI) for the cohort was 27 (±5.3) which is pre-obese according to World Health Organization (WHO) criteria [17]. Six of the twenty women used tobacco and eleven consumed at least one alcoholic beverage per week. Four individuals were pharmacologically controlled hypertensives while the remaining 16 volunteers were healthy. The aforementioned individuals were using prescription medications to control their hypertension. Despite the use of hypertensive medications, the salivary flow rates for the four individuals were within normal limits (1-2 ml/min.).

In comparison, the cancer cohort (n=36) exhibited a mean age of 53 (±10) years and was composed of 23 African American and 13 Caucasian women. The average body mass index (BMI) for the cohort was 31 (±8.2) which is category one (slightly obese) according to WHO standards [17]. Thirteen of the twenty women used tobacco and twelve consumed at least one alcoholic beverage per week. Sixteen individuals were hypertensive while 16 volunteers were healthy. This was significantly different from the healthy cohort at the $X^2 = 4.19$, $p<0.04$ level. Twenty individuals were using prescription medications while 12 did not use medications. This was significantly different from the healthy cohort at the $X^2 = 4.56$, $p<0.03$ level. There were four non-responders to the questionnaire in the cancer cohort.

The outcomes for the gynecological variables are also listed in Table 1. There were 13 premenopausal and seven postmenopausal women in the healthy group. Only two individuals used birth control pill while six were on hormone replacement therapy. Two individuals had total hysterectomies. On average, the healthy cohort experienced their first menses at 13 years of age with the first birth being 22 (±5.2) years of age. The group had 2.1 (±8.2) pregnancies per woman and experienced two live births.

Among the cancer cohort, there were 11 premenopausal and 24 postmenopausal women in the healthy group. This was significantly different from the healthy cohort at the $X^2 = 5.83$, $p<0.01$ level. Three individuals used birth control pill while six were on hormone replacement therapy. Nine individuals had total hysterectomies. The cancer cohort experienced their first menses at 13 years of age with the first birth being 20 (±4) years of age. The group had four (±2.8) pregnancies per woman and experienced four (±2.8) live births. The number of pregnancies and number of live births was significantly different from the healthy cohort at the U = 457, $p<0.004$ and U = 497, $p<0.001$ level respectively.

Analyte	Status	n	%Detect.	Mean	Median	Std. Dev.	Std. Err.	p value
IL-1β	healthy	6	30	44.49	8.98	79.81	32.58	n.s.
	cancer	18	50	24.47	10.87	35.80	8.44	
IL-6	healthy	13	65	14.51	8.01	17.16	4.76	n.s.
	cancer	20	56	16.49	16.63	9.69	2.17	
IL 8	healthy	18	90	334.30	177.56	457.80	107.90	U = 177, p<0.06
	cancer	27	75	302.60	107.97	778.30	149.80	
IL-10	healthy	7	35	12.96	10.40	7.13	2.25	**U = 44, p<0.04**
	cancer	15	42	13.32	5.47	24.51	6.33	
IFNγ	healthy	6	30	12.15	10.47	9.51	3.88	U =15, p<0.09
	cancer	9	25	8.12	4.70	7.47	2.49	
EGF	healthy	10	50	195.80	84.20	402.40	89.98	U = 274, p<0.09
	cancer	32	89	372.60	122.25	630.50	106.57	
MCP-1	healthy	7	35	161.10	136.65	136.67	33.15	**U = 217, p<0.05**
	cancer	36	100	284.90	234.84	265.78	44.30	
VEGF	healthy	10	50	128.83	131.22	90.07	24.07	n.s.
	cancer	23	64	128.76	73.61	122.63	25.57	
SWS	healthy	20	100	1.49	1.30	0.61	0.14	n.s.
	cancer	36	100	1.20	1.08	0.65	0.11	
TP	healthy	20	100	1.11	0.98	0.34	0.65	n.s.
	cancer	36	100	1.12	0.81	0.67	0.28	

Abbreviations: SWS = Stimulated Whole Saliva; TP = Total Protein; % detect. = Percent of population detected with analyte; Std. Dev. = Standard Deviation; Std. Err. = Standard Error of the Mean; n.s. = Not Significant; U = Mann-Whitney U.

Table 2: Descriptive statistics for cytokines across health status

Table 2 and Figure 1 represent the mean values for the panel of markers according to health status, i.e., healthy versus cancer. The mean values in Table 2 show that IL-1β (↓50%), IL-8 (↓10%) and IFNγ (↓23%) were down-regulated for the cancer cohort, albeit not statistically significant, they were lower than the corresponding levels in the heathy group suggesting a possible inhibitory or down-regulatory trend with respect to their concentration. Conversely, IL-10 (↑9%), EGF (↑53%) and MCP-1

(↑43%) were elevated among the cancer cohort suggesting an up-regulation of these cytokines. VEGF concentrations appeared unchanged. IL-10 and MCP-1 were significant at the U = 44, p<0.04 and U = 217, p<0.05 levels respectively. Differences in IL-8, IFNγ and EGF were not statistically significant, but were approximate to the p<0.05 alpha level. They were mentioned as they may not be statistically significant, but may be biochemically or clinically relevant [14].

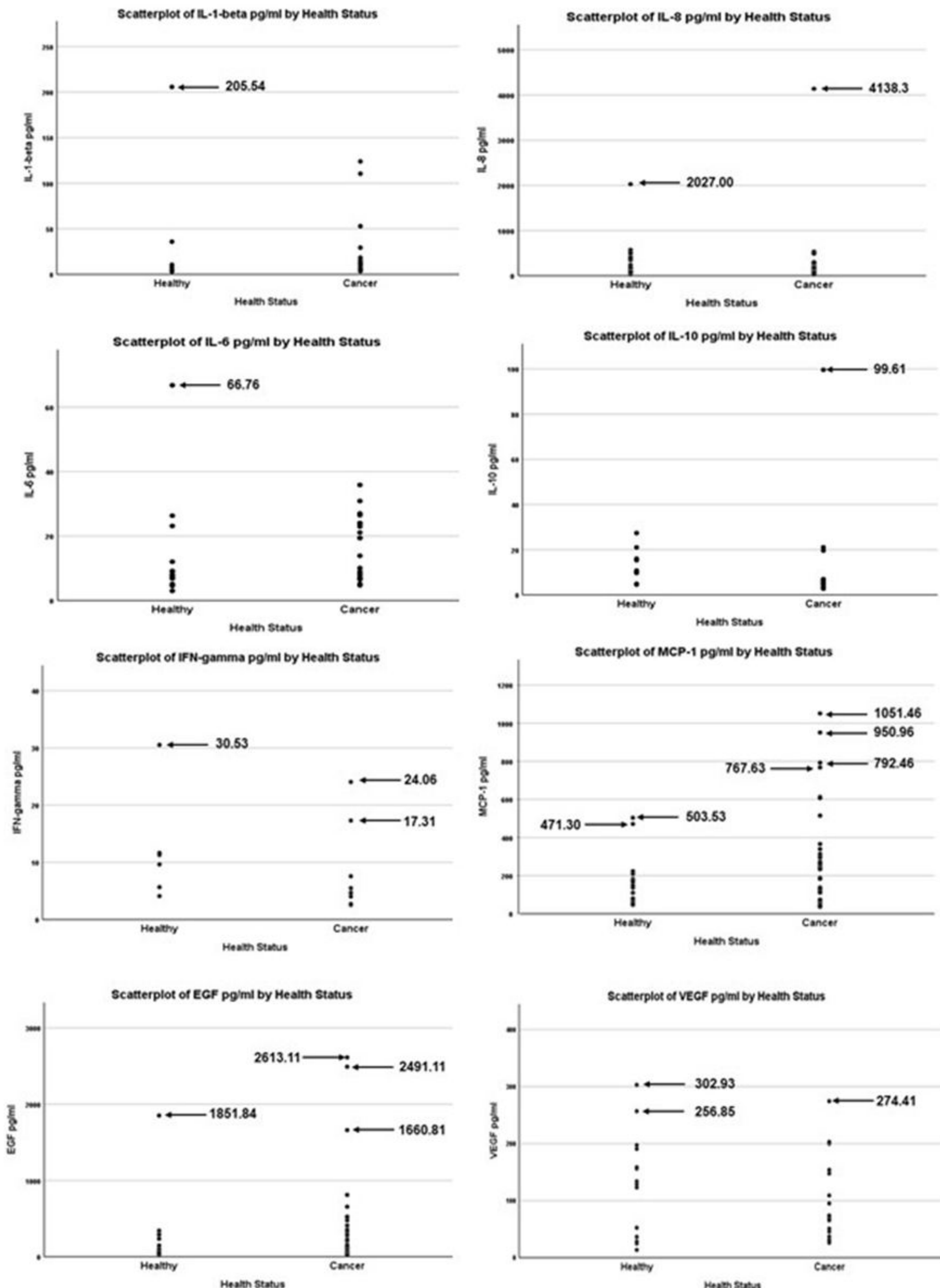

Figure 1: Scatterplot graphs of cytokine concentrations with outliers.

As illustrated in Figure 1, nearly all the analytes had outliers. Outliers were not removed from the analysis, which is represented in Table 2. However, when they outliers were removed and the analysis repeated, Il-1 β, IL-6, IL-8, IFNγ, EGF and VEGF did not change to a significant alpha level when the healthy and cancer cohorts were compared to each other. IL-10 and MCP-1, however, exhibited a slight increase in alpha level to $p<0.03$ and $p<0.04$ respectively.

The Spearman rho correlation coefficients (Table 3) were performed across the cytokine panel for both the healthy and the cancer cohorts. For the healthy cohort, IFNγ concentrations were strongly correlated with IL-10 ($\rho = 0.83$, $p<0.04$) and EGF ($\rho = -0.89$, $p<0.02$). EGF was not only correlated to IFNγ, but was also correlated with MCP-1 ($\rho = 0.60$, $p<0.01$) and VEGF ($\rho = 0.86$, $p<0.001$). As previously mention MCP-1 was correlated with EGF, but was also correlated with IL-1β ($\rho = 0.89$, $p<0.02$) and IL-8 ($\rho = 0.54$, $p<0.04$).

Analyte	Statistic	IL-1β	IL-8	IL-10	IFNγ	EGF	MCP-1	VEGF
IL-1β	Rho	.	0.29	-0.31	-0.26	0.45*	0.40	0.16
	Sig.	.	0.25	0.36	0.62	0.03	0.05	0.54
	n	.	17	11	6	24	24	18
IL-8	Rho	0.29	1.00	-0.10	-0.25	0.11	0.27	0.39*
	Sig.	0.25	.	0.67	0.49	0.48	0.09	0.05
	n	17.00	.	19	10	44	42	27
IL-10	Rho	-0.31	.	.	0.92**	0.12	0.30	0.24
	Sig.	0.36	0.67	.	0.00	0.56	0.17	0.36
	n	11	19	.	13	25	23	17
IFNγ	Rho	-0.26	-0.25	0.92**	.	0.10	0.46	0.29
	Sig.	0.62	0.49	0.01	.	0.71	0.10	0.31
	n	6	10	13	.	15	14	14
EGF	Rho	0.45*	0.11	0.12	0.10	.	0.67**	0.57**
	Sig.	0.03	0.48	0.56	0.71	.	0.00	0.00
	n	24	44	25	15	.	52	37
MCP-1	Rho	0.40	0.27	0.30	0.46	0.67**	.	0.56**
	Sig.	0.05	0.09	0.17	0.10	0.00	.	0.00
	n	24	42	23	14	52	.	34
VEGF	Rho	0.16	0.39*	0.24	0.29	0.57**	0.56**	.
	Sig.	0.54	0.05	0.36	0.31	0.00	0.00	.
	n	18	27	17	14	37	34	.

Abbreviations: Rho = Spearman Rho correlation coefficient * = Correlation is significant at the 0.05 level; **= Correlation is significant at the 0.01 level.

Table 3: Spearman Rho correlations for analytes across the cancer cohort

With respect to the cancer cohort, IFNγ concentrations were strongly correlated with IL-10 ($\rho = 0.92$, $p<0.01$). EGF was correlated with IL-1 β ($\rho = 0.45$, $p<0.03$), MCP-1 ($\rho = 0.67$, $p<0.001$) and VEGF. ($\rho = 0.57$ $p<0.01$). Additionally, MCP-1 was correlated with VEGF ($\rho = 0.56$, $p<0.01$). VEGF was correlated with IL-8 ($\rho = 0.39$, $p<0.05$), EGF ($\rho = 0.57$, $p<0.01$) and MCP-1 ($\rho = 0.57$, $p<0.01$).

Additionally, contingency correlations for nominal data were performed among the independent variables. Health status (healthy vs. cancer) was moderately correlated to menopausal status ($\rho = 0.33$, $p<0.02$) and the presence of systemic disease ($\rho = 0.29$, $p<0.04$). Menopausal status was also correlated to the presence of systemic disease ($\rho = 0.42$, $p<0.04$) while alcohol and tobacco usage were correlated at $\rho = 0.39$, $p<0.004$.

Table 4 indicates analyte concentrations across the various independent variables. Only one independent variable, alcohol usage, appeared to be influential. Those who consumed more than two drinks per week appeared to have lower EGF and MCP-1 concentrations than their non-drinking counterparts did. The MCP-1 concentration was significant at the $U = 11$, $p<0.02$ level.

The several independent variables among the cancer cohort were worth noting (Table 4). For example, tobacco usage exhibited a lowering of IL-8 levels ($p<0.06$). Once again, the finding, albeit not statistically significant, may be biochemically or clinically relevant [14].

Variable	Status	n	Mean	Std. Dev.	Std. Err.	U & p values
Healthy Cohort						
Alcohol Usage						
EGF	No	9	320.85	582.64	194.21	U=28, p<0.08
	Yes	11	93.56	102.46	30.89	
MCP-1	No	8	242.27	160.47	56.74	**U=11, p<0.02**
	Yes	9	88.97	48.82	16.27	
Cancer Cohort						
Tobacco Usage						
IL-8	No	16	439.23	996.90	249.23	U=50, p<0.06
	Yes	11	103.90	104.19	31.42	
Alcohol Usage						
IL-8	No	11	585.90	1189.10	358.53	**U=17, p<0.01**
	Yes	10	74.71	64.31	20.34	
MCP-1	No	14	405.25	297.56	79.53	**U=54, p<0.04**
	Yes	14	179.04	112.40	30.04	
Menopausal Status						
MCP-1	Pre - Meno.	11	238.14	316.26	95.36	U=181, p<0.09
	Post - Meno.	24	313.52	246.64	50.35	
On Medications						
VEGF	No	9	56.47	23.15	7.72	**U=86, p<0.02**
	Yes	12	153.57	122.09	35.24	

Abbreviations: Meno = Menopausal; n = sample size; Std. Dev. = Standard Deviation; Std. Err. = Standard Error of the mean; U = Mann-Whitney U.

Table 4: Statistics for independent variables vs. analytes

With respect to alcohol usage among the cancer group, both IL-8 and MCP-1 concentrations were significantly higher among non-tobacco users at the $p<0.01$ and $p<0.04$ alpha levels respectively

Table 4 also shows analyte concentrations across the various independent variables with respect to the use of prescription medications. As shown VEGF concentrations were significantly higher among prescription medications users at the $p<0.02$ alpha level.

The clinical pathology variables are illustrated in Tables 5 and 6. Table 5 presents the cytokine concentrations according to tumor staging and nodal status. There were 36 subjects in the cancer cohort. Their clinical staging was as follows: seven patients were categorized as Stage 0, eight as Stage I, ten as Stage IIa and eleven were Stage IIb. With respect to lymph node involvement, twenty-five were node negative while eleven were node positive. None of the individuals had distant metastases.

A comparison of analyte values across tumor size were not significant; however, tumor size greater than 2.0 cm exhibited a 50% increase for IL-6, 38% increase for MCP-1 and 84% increase for VEGF. Conversely, the same patients had an 18% decrease for IL-1β, 68% decrease for IL-8 and 67% decrease for Il-10, 19% decrease for EGF. There was insufficient amount of data for assessment of IFNγ with respect to tumor size.

Among those individuals with lymph node involvement IL-6, IL-8, IL-10, IFNγ, EGF and VEGF concentrations were lower than those that were lymph node negative. In contrast, Il-1β and MCP-1 were increased in concentration. IL-10 was significant at the $U = 3$; $p<0.03$ level, but the sample size was too small to be considered relevant and may be due to a type I error.

Nearly all cytokine concentrations with the exception of VEGF were increased among the ER positive patients. Similar findings were associated with the PR positive patients with the following exception: EGF was lower and VEGF was higher. The only statistically significant finding was that Her2/neu receptor negative cohort exhibited significantly higher IL-1β concentrations than the Her2/neu receptor positive counterparts did ($U = 28$; $p<0.05$).

As previously mentioned, eight subjects from the cancer cohort had saliva specimens taken one year after diagnosis and after their respective chemotherapy regimens were completed. Their specimens were assayed to determine if the analyte concentrations were modulated after therapy.

Analyte	Staging	n	Mean	Std. Dev.	Std. Err.	95% C.I.		Min.	Max.
						Lower	Upper		
IL-1β	Stage 0	3	39.45	61.45	35.48	-113.20	192.10	3.85	110.41
	Stage I	2	9.91	3.30	2.34	-19.76	39.57	7.57	12.24
	Stage IIa	7	10.09	9.13	3.45	1.64	18.53	3.56	29.30
	Stage IIb	6	38.61	44.68	18.24	-8.28	85.50	10.69	123.84
IL-6	Stage 0	2	21.73	3.30	2.34	-7.94	51.39	19.39	24.06
	Stage I	3	7.91	2.81	1.62	0.92	14.90	4.73	10.08
	Stage IIa	7	17.54	11.62	4.39	6.80	28.29	5.17	35.84
	Stage IIb	8	17.49	9.69	3.43	9.38	25.59	6.69	30.86
IL-8	Stage 0	7	758.28	1499.11	566.61	-628.16	2144.72	45.67	4138.30
	Stage I	7	130.14	159.94	60.45	-17.78	278.06	28.69	489.60
	Stage IIa	6	162.95	71.38	29.14	88.05	237.86	62.93	260.00
	Stage IIb	7	139.13	141.49	53.48	8.28	269.99	4.51	305.35
IL-10	**Stage 0**	**3**	**37.13**	**54.11**	**31.24**	**-97.30**	**171.55**	**5.86**	**99.61**
	Stage I	4	8.87	7.38	3.69	-2.87	20.61	2.75	19.60
	Stage IIa	5	8.57	7.03	3.14	-0.17	17.30	4.75	21.05
	Stage IIb	**3**	**3.38**	**0.64**	**0.37**	**1.78**	**4.98**	**2.90**	**4.11**
IFNγ	Stage 0	1	17.31	.	.	.	.	.	.
	Stage I	0	.	.	.	.	.	.	.
	Stage IIa	4	9.97	9.60	4.80	-5.32	25.25	2.73	24.06
	Stage IIb	4	3.98	1.01	0.50	2.37	5.59	2.54	4.70
EGF	Stage 0	7	466.17	910.84	344.26	-376.21	1308.55	20.72	2491.11
	Stage I	7	451.42	959.47	362.65	-435.94	1338.78	29.35	2613.75
	Stage IIa	10	334.14	494.55	156.39	-19.64	687.92	35.64	1660.81
	Stage IIb	11	297.78	251.08	75.70	129.11	466.46	50.92	810.33
MCP-1	Stage 0	7	361.55	399.11	150.85	-7.56	730.67	37.90	1051.48
	Stage I	8	142.87	94.72	33.49	63.68	222.05	35.66	300.91
	Stage IIa	10	330.08	263.68	83.38	141.46	518.71	61.66	950.96
	Stage IIb	11	298.46	245.57	74.04	133.49	463.44	38.92	767.63
VEGF	Stage 0	1	109.05	.	.	.	.	.	.
	Stage I	2	48.32	3.67	2.60	15.34	81.29	45.72	50.91
	Stage IIa	9	132.63	138.92	46.31	25.85	239.41	28.98	456.60
	Stage IIb	11	142.01	126.88	38.26	56.78	227.25	26.24	448.59
Tumor Size									
IL-1β	<2.0 cm	5	27.63	46.40	20.75	-29.98	85.24	3.85	110.41
	>2.0 cm	13	23.25	33.05	9.17	3.28	43.22	3.56	123.84
IL-6	<2.0 cm	6	12.30	7.68	3.13	4.24	20.35	4.73	24.06
	>2.0 cm	14	18.29	10.15	2.71	12.4	24.15	5.17	35.84
IL-8	<2.0 cm	16	419.55	1003.00	250.75	-114.91	954.01	28.69	4138.30
	>2.0 cm	11	132.53	110.29	33.25	58.43	206.62	4.51	305.35
IL-10	<2.0 cm	7	20.98	35.09	13.26	-11.47	53.43	2.75	99.61
	>2.0 cm	8	6.62	5.97	2.11	1.63	11.61	2.90	21.05
IFNγ	<2.0 cm	1	17.31	.	.	.	.	.	.
	>2.0 cm	8	6.97	7.09	2.51	1.05	12.90	2.54	24.06
EGF	<2.0 cm	16	414.73	845.51	211.38	-35.81	865.27	20.72	2613.75
	>2.0 cm	19	337.08	390.03	89.48	149.09	527.07	35.64	1660.81
MCP-1	<2.0 cm	17	237.75	275.43	66.80	96.14	379.36	35.66	1051.48
	>2.0 cm	19	327.16	256.77	58.91	203.39	450.92	38.92	950.96

Analyte	Staging	n	Mean	Std. Dev.	Std. Err.	95% C.I.		Min.	Max.
						Lower	Upper		
Tumor Size									
VEGF	<2.0 cm	5	77.71	52.21	23.35	12.9	142.54	28.29	153.90
	>2.0 cm	18	142.94	133.55	31.48	76.53	209.36	26.24	456.60
Lymph Node Involvement									
IL-1β	Node -	11	18.66	31.31	9.44	-2.38	39.69	3.85	110.41
	Node+	7	33.60	42.88	16.21	-6.06	73.26	3.56	123.84
IL-6	Node -	10	17.57	10.18	3.22	10.28	24.85	4.73	35.84
	Node+	10	15.42	9.60	3.04	8.55	22.28	6.61	30.86
IL-8	Node -	18	374.01	949.87	223.89	-98.35	846.37	28.69	4138.30
	Node+	9	159.81	129.97	43.32	59.91	259.71	4.51	305.35
IL-10	Node -	12	15.81	27.03	7.80	-1.37	32.98	2.75	99.61
	Node+	3	3.38	0.64	0.37	1.78	4.98	2.90	4.11
IFNγ	Node -	5	11.43	8.94	4.00	0.33	22.54	2.73	24.06
	Node+	4	3.98	1.01	0.50	2.37	5.59	2.54	4.70
EGF	Node -	22	439.40	771.95	164.58	97.14	781.66	20.72	2613.75
	Node+	13	259.49	247.59	68.67	109.87	409.11	35.64	810.33
MCP-1	Node -	23	280.24	291.22	60.72	154.30	406.17	35.66	1051.48
	Node+	13	293.25	224.57	62.28	157.55	428.96	38.92	767.63
VEGF	Node -	10	129.65	131.19	41.49	35.80	223.49	30.92	456.60
	Node+	13	128.08	121.06	33.58	54.92	201.24	26.24	448.59

Abbreviations: STD. Dev. = Standard Deviation; STD. Err. = Standard Error of the mean; C.I. = Confidence Interval; Min. = Minimum; Max. = Maximum; U = Mann-Whitney U.
Table 5: Descriptive statistics for staging and nodal status

Cytokine	ER Status	n	Mean	Std. Dev.	PR Status	n	Mean	Std. Dev.	Her2 Status	n	Mean	Std. Dev.
IL-1β	Negative	7	15.97	16.80	Negative	8	16.78	16.84	Negative	11	35.62*	42.67
	Positive	9	35.19	47.26	Positive	8	36.79	49.90	Positive	5	7.35	3.79
IL-6	Negative	7	10.85	7.57	Negative	8	13.96	11.29	Negative	13	14.36	10.39
	Positive	9	18.18	11.35	Positive	8	15.98	9.84	Positive	3	17.60	11.53
IL 8	Negative	7	178.33	163.52	Negative	10	149.42	137.99	Negative	12	457.24	1162.64
	Positive	12	441.34	1166.81	Positive	9	561.13	1344.80	Positive	7	151.07	162.69
IL 10	Negative	4	8.09	7.78	Negative	5	7.84	6.70	Negative	8	18.21	33.34
	Positive	10	16.06	29.82	Positive	9	17.08	31.44	Positive	6	7.88	6.61
IFN-γ	Negative	2	4.34	0.45	Negative	2	6.12	2.06	Negative	6	6.47	5.61
	Positive	6	9.95	8.78	Positive	6	9.36	9.09	Positive	2	14.78	13.13
EGF	Negative	11	377.56	752.53	Negative	13	456.43	779.65	Negative	17	531.25	853.48
	Positive	14	434.03	725.44	Positive	12	358.00	685.53	Positive	8	149.78	105.31
MCP-1	**Negative**	**11**	**245.63**	**240.69**	**Negative**	**14**	**249.96**	**207.75**	**Negative**	**17**	**289.54**	**249.29**
	Positive	**15**	**271.14**	**197.02**	**Positive**	**12**	**272.46**	**226.13**	**Positive**	**9**	**205.22**	**106.47**
VEGF	**Negative**	**8**	**89.73**	**83.68**	**Negative**	**8**	**84.72**	**86.26**	**Negative**	**14**	**81.65**	**64.95**
	Positive	**11**	**89.54**	**61.01**	**Positive**	**11**	**93.19**	**58.16**	**Positive**	**5**	**111.93**	**83.70**

Abbreviations: *Her2+ < Her2-; Mann-Whitney U = 28.0; $p<0.05$; ER = Estrogen Receptor Status; PR = Progesterone Receptor status; Her2 = Her2/*neu* Receptor Status
Table 6: Cytokine means and standard deviations across receptor status

Figures 2a-2g display the changes in analyte mean concentrations post treatment. These figures provide the analyte concentrations, tumor staging, receptor status and treatment regimen for the seven individuals. There were two Stage IIa (Patients 1-3) and five Stage IIb (Patients 4-7) individuals. One individual died shortly after the chemotherapy was completed. IL-10, MCP-1 and EGF exhibited the largest decrease among the cytokines. IL-10 and IFNγ are not shown due to missing data in either pre or post-treatment conditions.

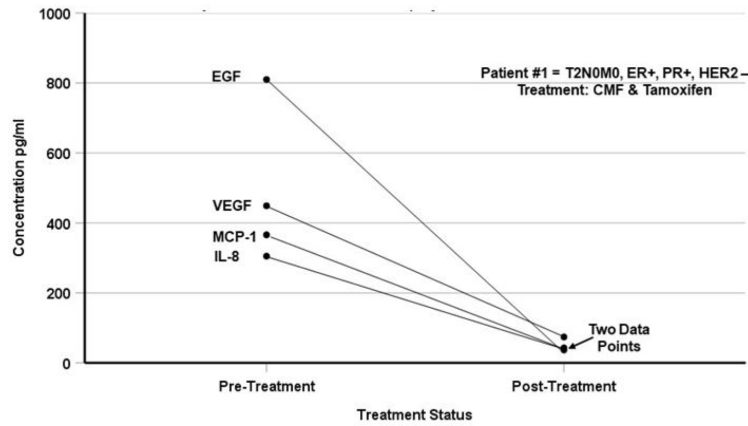

Figure 2a: Patient #1 pre and post-treatment cytokine concentrations.

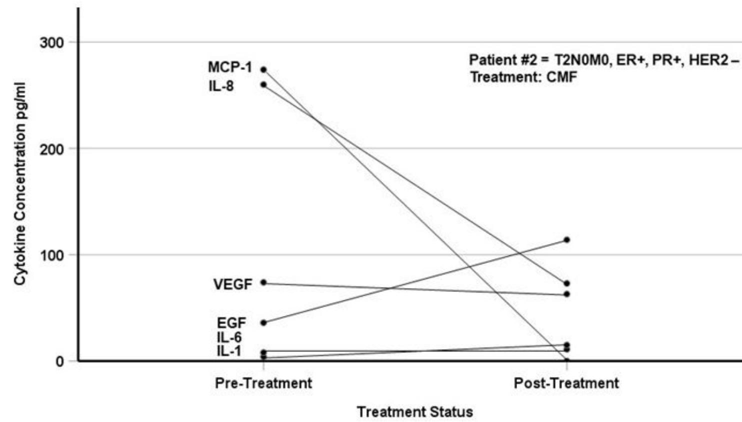

Figure 2b: Patient #2 pre and post-treatment cytokine concentrations.

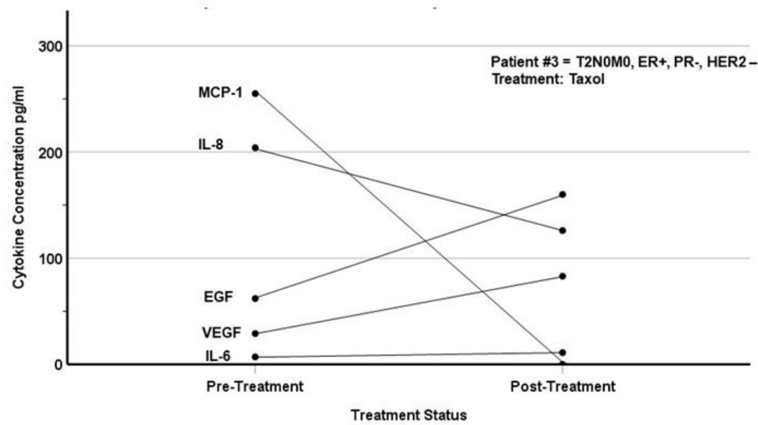

Figure 2c: Patient #3 pre and post-treatment cytokine concentrations.

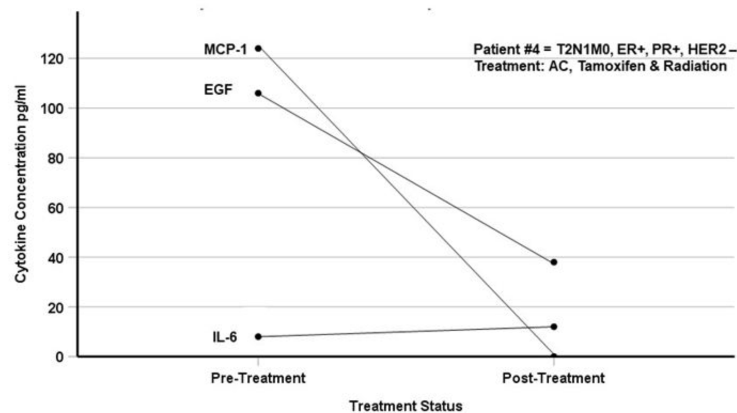

Figure 2d: Patient #4 pre and post-treatment cytokine concentrations.

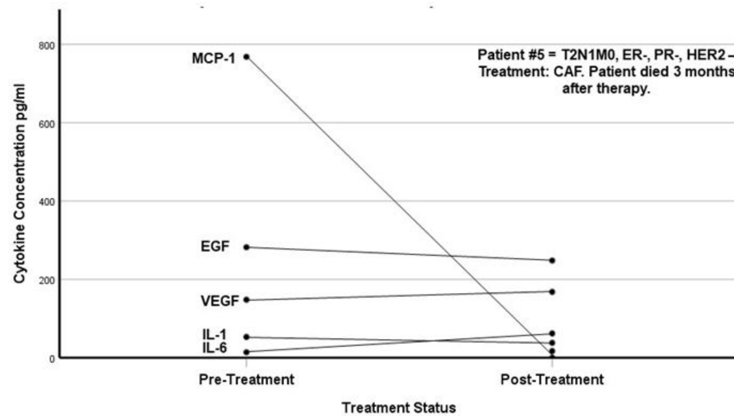

Figure 2e: Patient #5 pre and post-treatment cytokine concentrations.

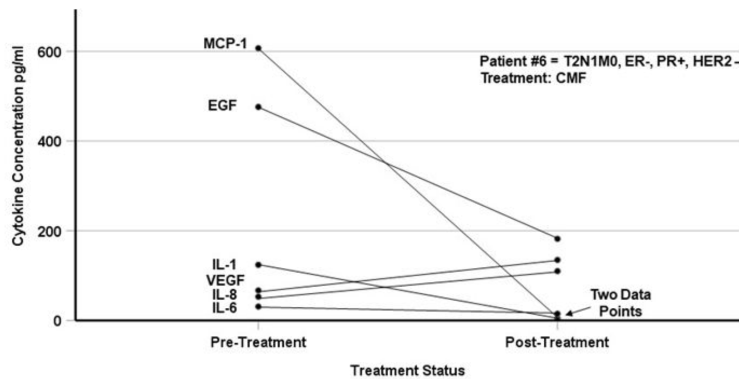

Figure 2f: Patient #6 pre and post-treatment cytokine concentrations.

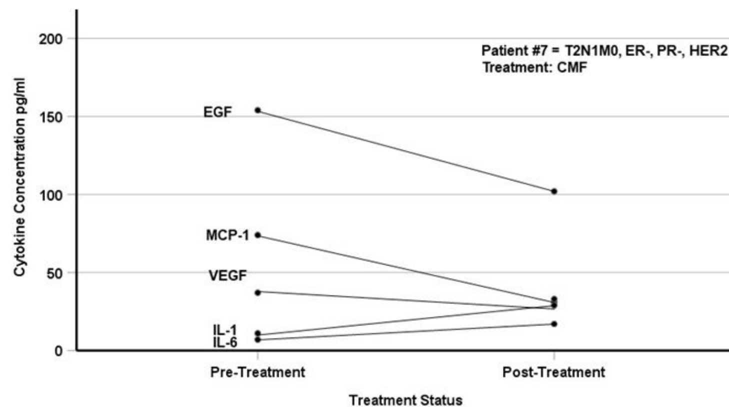

Figure 2g: Patient #7 pre and post-treatment cytokine concentrations.

Discussion

To the best of our knowledge, this study is the first to profile a spectrum of various cytokines, chemokines and growth factors in the saliva of breast cancer patients. There is a paucity of prior research to compare the results of this study. Consequently, the authors will compare the results to those assessing the analytes in different media (e.g., serum, cell supernatants, etc.).

As previously stated, the main objectives of the present study are to: (i) identify and compare the abundance of cytokines present in the saliva of breast cancer and a healthy population and identify any possible correlations between cytokines present in saliva among healthy and diseased population and (ii) characterize the cytokine profiles of various tumor subtypes.

As shown in Table 2, IL-10 and MCP-1 were significantly elevated among the cancer group at the $p<0.04$ and $p<0.05$ alpha levels, respectively. The elevation of IL-10 in this study is supported by Espinoza *et al.* which found increased levels of IL-10, a molecule with immunosuppressive and immune-stimulatory properties, in the tumor interstitial fluid of breast cancer patients [17]. MCP-1 was found in the plasma of gastric cancer patients and was correlated with poor prognosis [18-20].

Similar to the results reported by Velazquez et al., IL-10 is upregulated among the ER/PR positive patients in this study as well

[18]. As shown in Table 6, IL-10 concentrations were nearly doubled among ER/PR positive patients; however, the sample size is small and requires further evaluation using a larger sample size.

Likewise, MCP-1, also known as CCL2 was also significantly upregulated. These results agree with the findings of other studies investigating the presence of MCP-1 secondary to carcinoma [21-24]. MCP-1 is a chemokine with potent monocyte activating protein, may mediate the migration of monocytes from the circulation to breast tumors. It is also associated with angiogenesis and breast tumor progression [24,25]. As shown in Table 3, MCP-1 is also strongly associated with IL-1β, IL-8, EGF and VEGF, which are likewise involved with angiogenesis and breast tumor progression [24,25]. In summary, all the significant cytokines presented in Table 3 appear to be associated with a strong pro-inflammatory response secondary to the presence of carcinoma of the breast. The increase of IL-10 may be the anti-inflammatory response to the pro-inflammatory cytokines.

In Figures 2a – 2g, there was no set pattern as to the changes in concentration among post-treatment patients. Only MCP-1 decreased in all seven cases. This may be due to a partial response to treatment unlike Patient #1 where all the cytokines that were tested decreased. In these instances, those patients may have been in clinical remission, but not in molecular remission. Of particular interest were the outcomes for Patient #5. With the exception of MCP-1, Patient #5's analyte concentrations remained unchanged suggesting that the individual did not respond to therapy and progressed to terminal disease.

Conclusion

The exacerbated production and secretion of cytokines and growth factors by cancer cells and tumor-infiltrating immune cells is a consistent feature of breast cancer tissues. Here, the authors provide evidence, albeit preliminary, that tumor-infiltrating lymphocytes and monocytes are contributors to the total pool of secreted cytokines, which in combination with the leakage of tumor-produce circulating cytokines into the bloodstream may account for the higher or lower concentrations of certain cytokines in the saliva of breast cancer patients. Consequently, further studies are needed to confirm and address the biological and clinical relevance of salivary cytokines in relation to human breast cancer.

Acknowledgments

The research presented in this manuscript was supported by the Avon Breast Cancer Foundation (#07-2007-071), Komen Foundation (KG080928), Gillson-Longenbaugh Foundation and the Texas Ignition Fund.

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