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The Medical Oncology Centre of Rosebank
Personalised Cancer Care

Dysregulation of immune checkpoint proteins in newly- diagnosed early breast cancer patients

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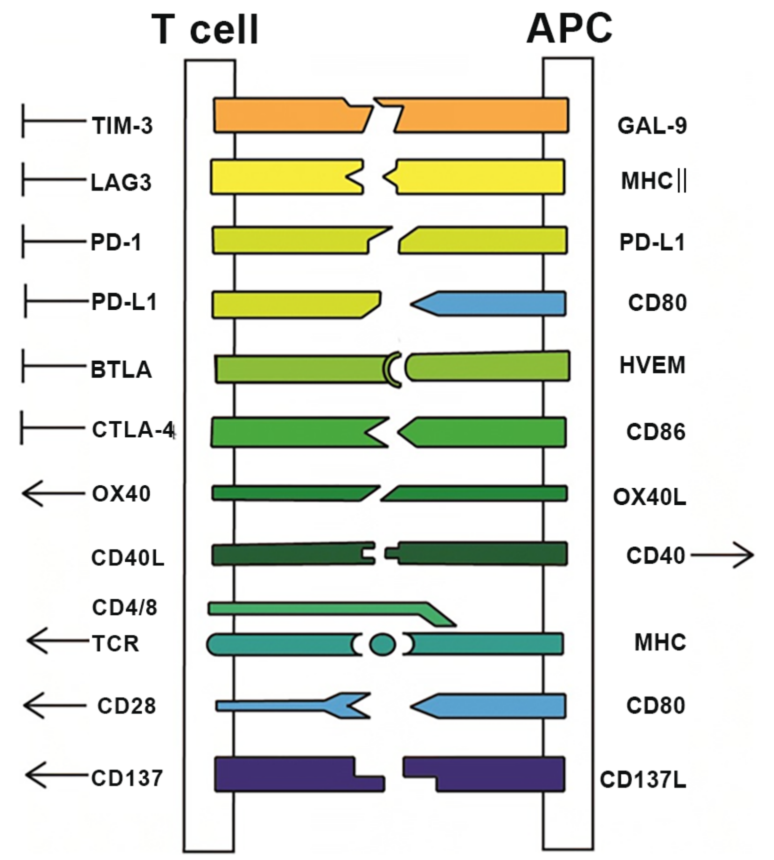
Background

- For effective killing of cancer cells in an anticancer immune response, a series of events involving different immune cells needs to be initiated and allowed to proceed. The steps in the cancer immunity cycle start with the release of tumor cell antigens, which are recognized by and lead to the killing of cancer cells by cytotoxic T cells. This immune response is modulated by a variety of stimulatory and inhibitory factors;
- T cells need two signals for activation: binding of the TCR (T-cell receptor) to the MHC (major histocompatibility complex) and activation of co-stimulatory molecules;
- Immune checkpoints can stimulate or inhibit these events thereby regulating the functions of immune cells;
- Accordingly, checkpoints play important roles in the maintenance of immune homeostasis;
- Examples of stimulatory molecules include TCR/MHC, CD137L/CD137 and OX40L/CD40, while CTLA-4/CD80 or CD86 and PD-1/PD-L1 are potent inhibitory checkpoints. Increasing numbers of novel regulatory receptors and ligands have recently been described and are summarized in figure 1;
- Recently, a series of soluble systemic immune checkpoints such as sCTLA-4 (soluble CTLA-4), sPD-1 (soluble PD-1) and others have been identified that can be measured in plasma.

Figure 1. Stimulatory and inhibitory immune checkpoint molecules.



Costimulatory:
CD28 Costimulatory immune checkpoint molecule
HVEM (Herpes Virus Entry Mediator)
Costimulatory immune checkpoint molecule
CD80 (B7-1) Ligand of stimulatory CD28
CD137 Costimulatory immune checkpoint molecule
CD27 Costimulatory immune checkpoint molecule
GITR (Glucocorticoid –induced TNFR-related protein)
Costimulatory immune checkpoint molecule
GITRL (Glucocorticoid –induced TNFR-related protein ligand)
Costimulatory immune checkpoint molecule
CD86 Costimulatory immune checkpoint molecule
CD40 Costimulatory immune checkpoint molecule
ICOS (Inducible T cell costimulator)
Costimulatory immune checkpoint molecule

Co-inhibitory:
CD80 (B7-1) Ligand of inhibitory CTLA-4
HVEM (Herpes Virus Entry Mediator)
Co-inhibitory immune checkpoint molecule
CTLA-4 (Cytotoxic T lymphocyte-associated protein 4)
Co-inhibitory immune checkpoint molecule
PD-1 (Programmed cell death protein 1)
Co-inhibitory in T cell activation and cancer cell killing
PD-L1 (Programmed cell death protein 1 ligand) ligand
BTLA (B- and T-lymphocyte attenuator) HVEM ligand
LAG-3 (Lymphocyte Activating Gene 3)
Negatively regulates proliferation, activation, and homeostasis of T cells
TIM-3 (T cell Immunoglobulin and mucin-domain containing protein 3)
Immune checkpoint, regulates macrophage activation

Reference
Gu, D., Ao, X., Yang, Y. et al. Soluble immune checkpoints in cancer: production, function and biological significance. j. immunotherapy cancer 6, 132 (2018). https://doi.org/10.1186/s40425-018-0449-0

Methods

Aim

- The circulating levels of 16 immune checkpoint-related proteins panel (BTLA, GITR, GITRL, HVEM, LAG-3, PD-1, PD-L1, TIM-3, CD27, CD28, CD80, CD86, CD40, CD137, ICOS, TLR-2 and CTLA-4), as well as chemokines (CXCL5, CCL26, CX3CL1, CXCL10, CXCL9, CCL23) and cytokines (IL2, IL4, IL6, IL8, IL10, IL16, IL17A, IL1RA, Interferon α, Interferon γ TGF β1) were profiled in 98 early breast cancer patients (patient characteristics are summarized in table 1) and compared to those of 45 healthy controls.

Lab Method

- Plasma levels of immune-oncology checkpoints, chemokines and cytokines were assayed using Bio-Plex Suspension Bead Array platforms (Milliplex® or Bio-Rad® human magnetic bead panels). The methods were followed according to the manufacturers specifications and the data analysed using Bio-Plex Manager software 6.0 and results reported as either ng/mL or pg/mL. C-reactive protein (CRP) levels were determined by nephelometry using the *Cardio*Phase® *hs*CRP assay (Siemens Healthcare Diagnostics). The method was followed according to the manufacturer's instructions. Results are reported as µg/mL.

Statistical Methods

- The primary hypothesis was that there was a significant difference in the plasma levels of soluble immune checkpoints, cytokines and chemokines between breast cancer patients and healthy controls. Descriptive statistics were used to tabulate patient characteristics. The Mann Whitney U-test was used to compare levels of the various test biomarkers between breast cancer patients and healthy controls. Fisher's exact or Chi-squared tests were used for the analysis of categorical variables. NCSS software version 11 for Windows (USA) was used for statistical analyses.

Results

- Patient characteristics are shown in table 1. Comparison of plasma levels of immune checkpoints, chemokines, and cytokines between breast cancer patients and healthy controls are shown in Table 2.

Table 1. Patient Characteristics.

Age (n=98)	
Median Age	52
Range	27-85
Menopausal Status	
Peri-menopausal	2 (2%)
Pre-menopausal	55 (56%)
Post-menopausal	41 (42%)
Biological Type	
Her2 positive	16 (16%)
Luminal A	2 (2%)
Luminal B	14 (14%)
TNBC	66 (68%)
Grade	
1	1 (1%)
2	25 (26%)
3	69 (70%)

Stage	
1a	13 (13%)
2a	40 (41%)
2b	31 (32%)
3a	7 (7%)
3b	4 (4%)
3c	3 (3%)
Glands	
Negative	46 (47%)
Positive	52 (53%)
Estrogen Status	
Negative	67 (68%)
Positive	31 (32%)
Progesterone Status	
Negative	81 (82%)
Positive	17 (17%)
Her2 Status Status	
Negative	82 (84%)
Positive	16 (16%)
Ki-67 mean = 50% [6-100%]	
15 - 39%	31 (32%)
≤ 14%	7 (7%)
≥ 40%	58 (61%)

Table 2. Immune Checkpoint Molecule.

		Breast Cancer (n=98)			Controls (n=45)			
	Immune Checkpoint Molecule	Median	95% LCL of Median	95% UCL of Median	Median	95% LCL of Median	95% UCL of Median	p value
Co-stimulatory	CD27 pg/mL	3131,285	2639,21	3568,54	4577,35	3391,13	5784,85	0,00041
	CD28 pg/mL	32176,41	27889,65	3568,54	46135,18	27210,29	67544,1	0,00229
	CD40 pg/mL	1464,685	1262,67	1620,9	1977,68	1404,82	2569,56	0,00054
	ICOS pg/mL	14364,95	11122,68	15964,4	26506,65	15897,52	31725,99	0,0001
	GITR pg/mL	5529,8	4868,15	6407,6	7151,12	5528,36	9878,41	0.00234
	GITRL pg/mL	5529,8	4868,15	6407,6	7151,12	5528,36	9878,41	0,00234
Co-inhibitory	PD-1 pg/mL	11571,18	10147,12	13426,83	14917,48	7874,92	21795,02	0,11977
	PD-L1 pg/mL	1580,695	1198,87	1978,97	3342,62	2628,64	4750,96	0.0000
	CTLA-4 pg/mL	1585,73	1330,19	1790,69	2618,23	1578,44	3110,47	0,0006
	CD80 pg/mL	1613,265	1317,61	1792,55	2329,77	1395,01	3042,87	0,00108
	CD86 pg/mL	11199,42	9447,21	12851,98	14297,09	9391,46	20525,14	0,011
	TIM-3 pg/mL	3834,44	3436,22	4132,4	5046,87	4732,72	5958,87	0,00069
	LAG-3 pg/mL	120377,5	93854,44	138811,3	150416	94508,53	187997,2	0,11396
	BTLA pg/mL	12907,97	11108,41	17084,76	12907,97	11108,41	17084,76	0,18276
Chemokines	ENA 78 CXCL5 pg/mL	535,58	250,7	763,49	2246,51	1540,24	3246,49	0,00002
	Eotaxin 3 CCL26 pg/mL	4,31	4,31	8,41	6,36	3,28	8,41	0,35476
	Fractalkine CX3CL1 pg/mL	445,13	399,04	489,3	397,12	366,07	431,69	0,00871
	IP-10 CXCL10 pg/mL	485,82	426,98	607,59	543,33	498,35	638,22	0,86849
	MIG CXCL9 pg/mL	91,305	76,65	112,33	92,92	74,64	117,5	0,39967
	CCL23 pg/mL	535,58	250,7	763,49	2246,51	1540,24	3246,49	0,00002
Cytokines	IL-2 pg/mL	9,01	8,19	10,37	9,81	7,29	11,5	0,32947
	IL- 4 pg/mL	126,24	102,57	156,37	146,78	113,16	200,93	0,1878
	IL-6 pg/mL	10,52	8,9	11,3	10,4	7,56	13,71	0,74975
	IL-8 pg/mL	9,61	8,18	10,87	9,81	7,49	10,34	0,20475
	IL-16 pg/mL	1931,855	1569,84	2087,37	3535,39	2932,85	3813,73	0,00005
	Interferon α pg/mL	174,275	152,73	192,52	199,64	176,29	214,94	0,00751
	IL-1 Ra pg/mL	418,735	346,33	466,68	503,33	448,08	625,49	0,01766
	Interferon γ pg/mL	59,74	51,01	66,56	69,55	44,35	80,45	0,08564
	IL-10 pg/mL	42,76	36,5	50,87	47,61	34,16	59,25	0,40669
	IL-17A pg/mL	23,055	20,19	25,9	23,92	20,84	28,38	0,96121
	TGF β1 pg/mL	20353,26	14180,32	24904,45	23785,83	16184,42	36390,72	0,98696
Other	TLR-2 pg/mL	24059.42	20551,28	28354,07	30477.2	20928,44	50302,64	0.01406
	HVEM pg/mL	1866,92	1674,84	2007,57	2290,19	2079,46	2618,44	0,00001

Figure 2. GITR Breast Cancer vs. Control.

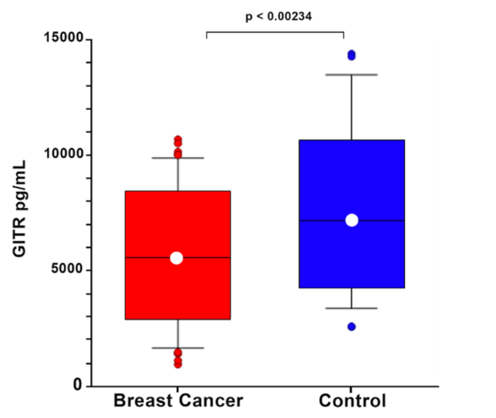


Figure 3. CD27 Breast Cancer vs. Control.

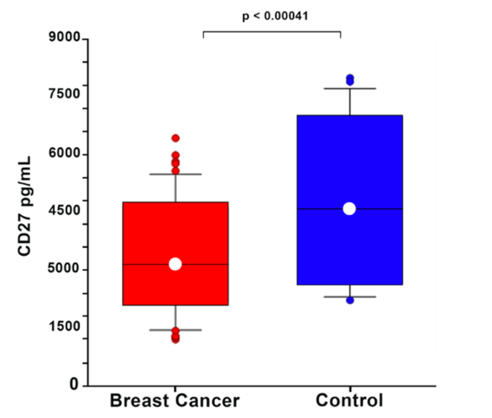


Figure 4. CD28 Breast Cancer vs. Control.

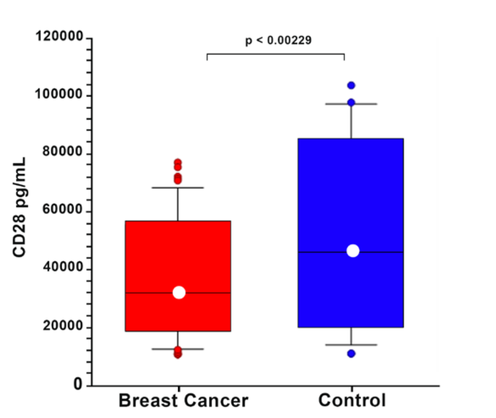


Figure 5. CD40 Breast Cancer vs. Control.

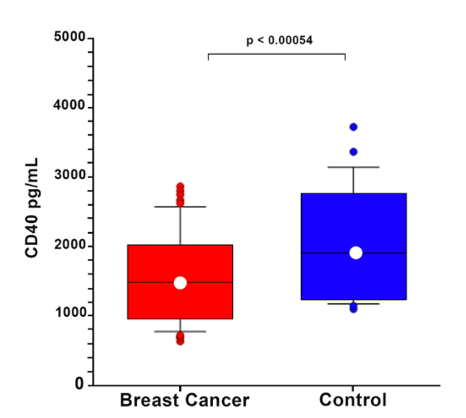


Figure 6. CD80 Breast Cancer vs. Control.

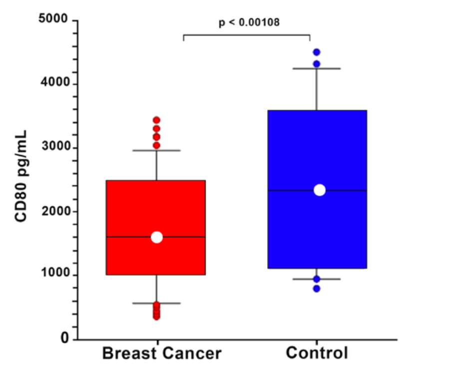


Figure 7. ICOS Breast Cancer vs. Control.

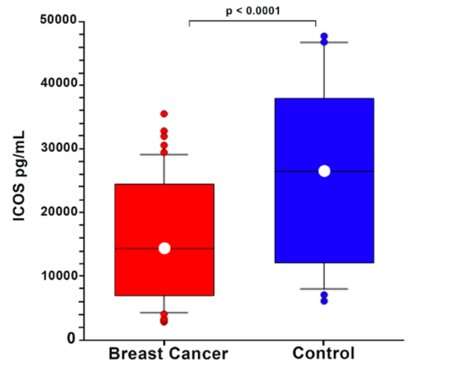


Figure 8. PD-L1 Breast Cancer vs. Control.

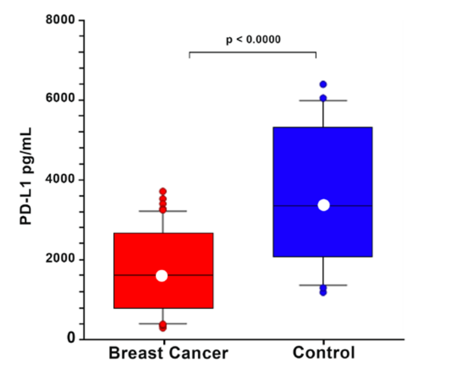


Figure 9. CTLA-4 Breast Cancer vs. Control.

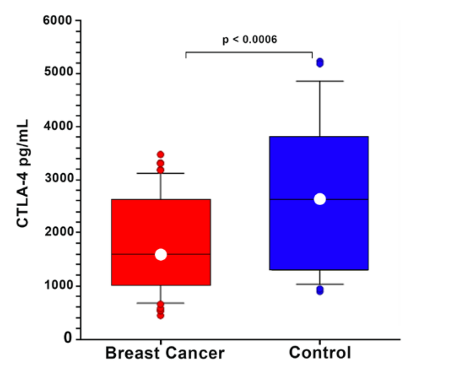


Figure 10. TIM-3 Breast Cancer vs. Control.

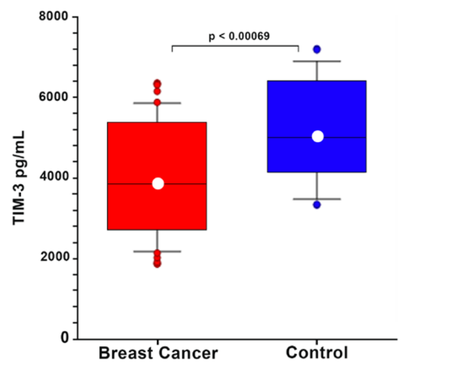


Figure 11. HVEM Breast Cancer vs. Control.

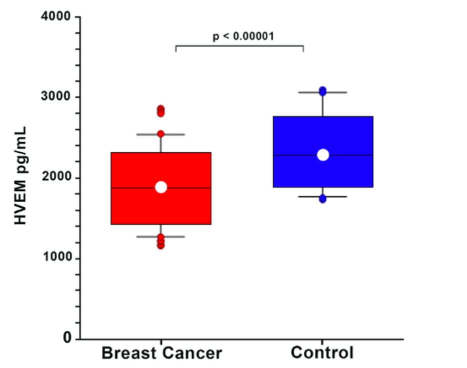


Figure 12. TLR-2 Breast Cancer vs. Control.

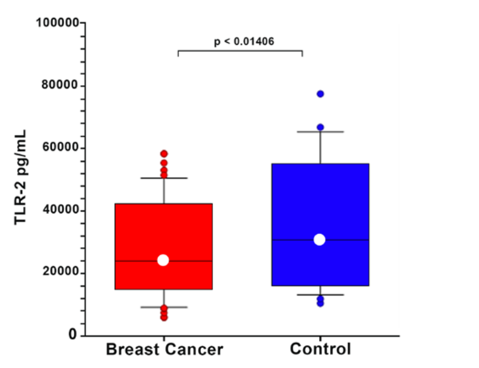


Figure 13. CXCL5 Breast Cancer vs. Control.

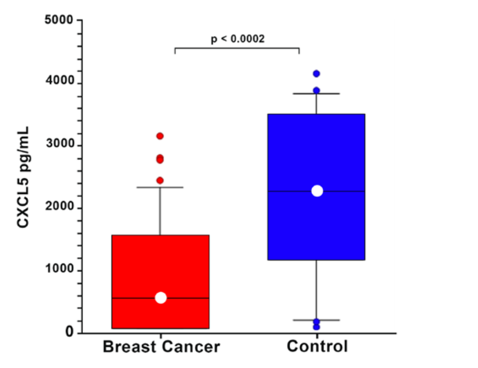


Figure 14. CCL23 Breast Cancer vs. Control.

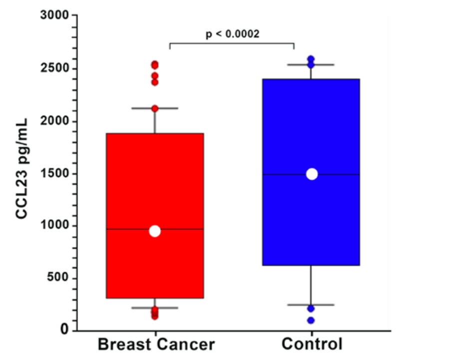


Figure 15. IL-16 Breast Cancer vs. Control.

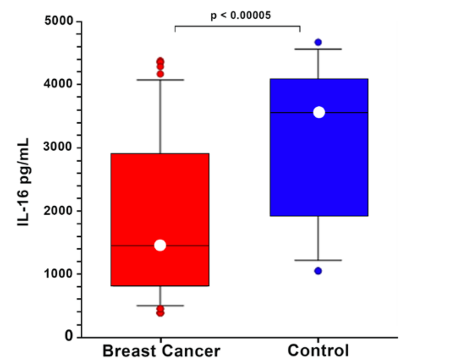


Figure 16. Interferon α Breast Cancer vs. Control.

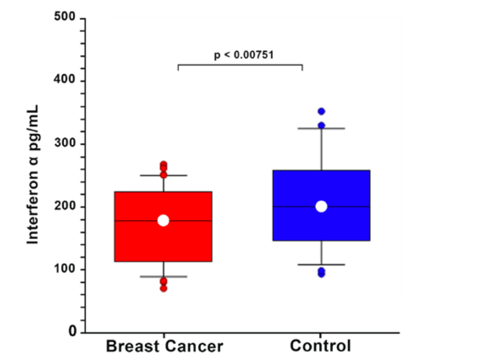


Figure 17. IL-1 Ra Breast Cancer vs. Control.

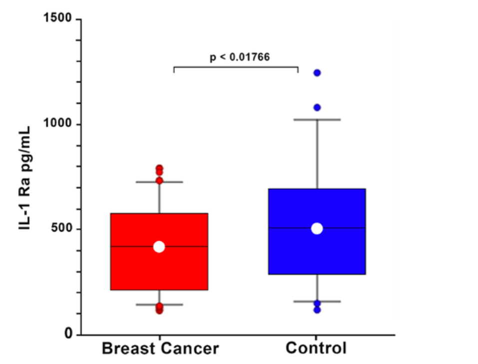


Figure 18. CX3CL1 Breast Cancer vs. Control.

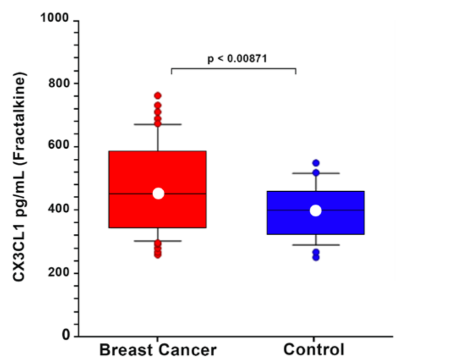


Figure 19. ENA 78 CXCL5 Breast Cancer vs. Control.

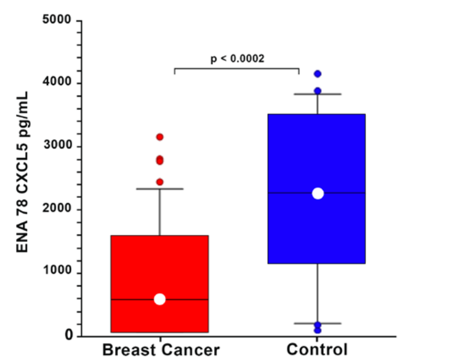


Figure 20. GITRL Breast Cancer vs. Control.

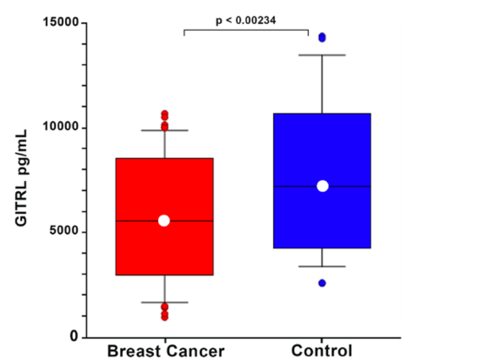
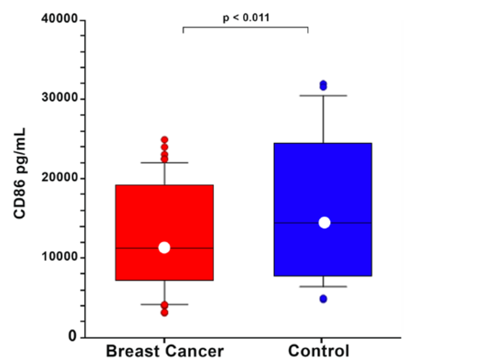


Figure 21. CD86 Breast Cancer vs. Control.



Conclusions

- Lower levels of a number of soluble costimulatory (n=6/6) and co-inhibitory (n=7/9) immune checkpoints, as well as chemokines (n=2/6) and cytokines (n=3/11), were identified in newly-diagnosed, non-metastatic breast cancer patients compared to healthy controls.
- These results indicate that early breast cancer is associated with a down-regulation of both stimulatory and inhibitory immune-checkpoint pathways. Newly- diagnosed early breast cancer patients appear to have a generalized immune-suppression independent of subtype and stage, which, to our knowledge, is the first study to simultaneously describe soluble immune checkpoints in early breast cancer patients.

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