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

BL Rapoport [1-2], HC Steel [1], S Nayler [3], C Benn [4], T Smit [2], L Heyman [1-2], AJ Theron [1], TN Hlatshwayo [1], LLI Kwofie [1], R. Anderson [1]

[1] *Department of Immunology, Faculty of Health Sciences, University of Pretoria, corner Doctor Savage Road and Bophelo Road, Pretoria 0002, South Africa.*

[2] *The Medical Oncology Centre of Rosebank, 129 Oxford Road, Saxonwold 2196, Johannesburg, South Africa.*

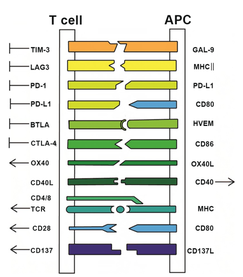
[3] *Drs Gritzman & Thatcher Inc Laboratories & Wits Donald Gordon Medical Centre, 4 Main Street, Bordeaux, Randburg, South Africa.*

[4] *Netcare Breast Care Centre, 1 Jan Smuts Ave, Johannesburg, South Africa.*

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 checkpoint molecules (ICM) 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.



Co-stimulatory immune checkpoint molecules
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 immune checkpoint molecules
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).

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, ICOS, TLR-2 and CTLA-4) were profiled in 75 early breast cancer patients (patient characteristics are summarized in table 1) and compared to those of 45 healthy controls.

Laboratory Method

- Plasma levels of immune-oncology checkpoints 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 pg/mL.

Statistical Methods

- The primary hypothesis was that that there was a significant difference in the plasma levels of soluble immune checkpoints between early breast cancer patients' pre-treatment, post-neoadjuvant chemotherapy (NAC), and post-surgery.
- Data was prospectively obtained, and levels compared between pre-treatment, post-NAC, post-surgery, and healthy controls using non-parametric tests (Mann-Whitney & Kruskal-Wallis).
- 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. P < .05 was considered statistically significant.
- NCSS software version 11 for Windows (USA) was used for statistical analyses.

Results

- Patient characteristics are shown in table 1.

Table 1. Patient Characteristics.

Age	
Median Age	54
Range	29-85
Menopausal Status	
Peri-menopausal	46 (64%)
Pre-menopausal	25 (35%)
Post-menopausal	1 (1%)
Grade	
1	1 (1%)
2	20 (28%)
3	49 (68%)
Unknown	2 (3%)
Tumor Size	
T1	21 (29%)
T2	42 (58%)
T3	6 (8%)
T4	3 (4%)
Nodal Status	
Positive	36 (50%)
Negative	36 (50%)
Stage	
1	12 (17%)
2A	32 (44%)
2B	20 (28%)
3	8 (11%)
Biological Type	
Her-2 Positive	10 (14%)
Luminal A	1 (1%)
Luminal B	9 (13%)
TNBC	51 (71%)
TNBC & Luminal B	1 (1%)
Ki-67	
≤ 14 %	3 (4%)
15 - 39%	23 (32%)
≥ 40%	45 (63%)
Unknown	1 (1%)

Table 2. Pathological complete response for the entire patient cohort and by biological type.

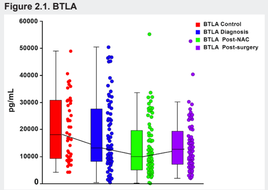
Pathological Complete Response (pCR)	
Yes	44 (61,11%)
No	28 (38,89%)
pCR by Biological Type	
Her-2 Positive	
Yes	8 (80%)
No	2 (20%)
Luminal	
Yes	3 (30%)
No	7 (70%)
TNBC	
Yes	33 (65%)
No	18 (35%)
TNBC & Luminal	
Yes	0 (0%)
No	1 (100%)

Table 3. The effect of treatments on soluble, systemic ICMs

ICM	Control	Diagnosis (Pre-Chemotherapy)	Post-NAC (Post-NAC)	Post-surgery (Post-Surgery)
	Median (pg/ml)			
BTLA	18147	13022	9987	12777
CD80	2329	1678	3048	3611
CD86	14297	11585	9922	12439
CTLA-4	2618	1566	598	687
LAG-3	150416	131275	464880	500133
PD-L1	3342	1647	4794	5215
PD-1	14917	12305	13350	15076
TIM-3	5047	3897	9975	9615
CD27	4577	3342	5351	5427
CD28	46135	32914	44277	50058
CD40	1977	1523	2030	2054
GITR	3797	1497	4035	4434
GITRL	7151	5886	5339	5927
ICOS	26506	15123	26586	29746
HVEM	2290	1865	4047	3950
TLR-2	30477	26831	33837	37042

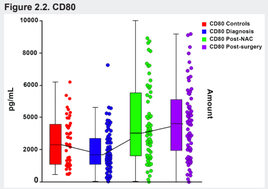
Figure 2. Comparison of ICM's between breast cancer patients at diagnosis, post-NAC, post-surgery and a control group.

Figure 2.1. BTLA



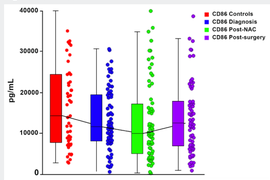
BTLA Comparisons	p-value
Control vs Pre-Treatment	0,23494
Control vs Post-Chemotherapy	0,00081
Control vs Post-Surgery	0,06832
Pre-Treatment vs Post -Chemotherapy	0,03655
Pre-Treatment vs Post -Surgery	0,32174
Post Chemotherapy vs Post-Surgery	0,23222

Figure 2.2. CD80



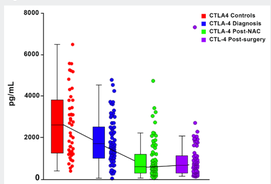
CD80 Comparisons	p-value
Control vs Pre-Treatment	0,07346
Control vs Post-Chemotherapy	0,02099
Control vs Post-Surgery	0,00451
Pre-Treatment vs Post -Chemotherapy	0,00001
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,64588

Figure 2.3. CD86



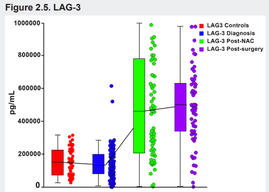
CD86 Comparisons	p-value
Control vs Pre-Treatment	0,17339
Control vs Post-Chemotherapy	0,04489
Control vs Post-Surgery	0,19755
Pre-Treatment vs Post -Chemotherapy	0,27890
Pre-Treatment vs Post -Surgery	0,82606
Post Chemotherapy vs Post-Surgery	0,22757

Figure 2.4. CTLA-4



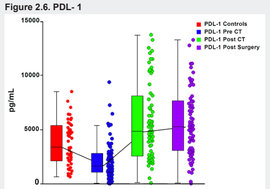
CTLA-4 Comparisons	p-value
Control vs Pre-Treatment	0,00792
Control vs Post-Chemotherapy	0,00000
Control vs Post-Surgery	0,00000
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,82918

Figure 2.5. LAG-3



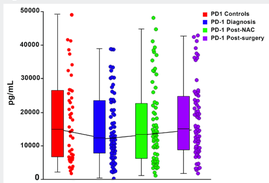
LAG-3 Comparisons	p-value
Control vs Pre-Treatment	0,58683
Control vs Post-Chemotherapy	0,00000
Control vs Post-Surgery	0,00000
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,59919

Figure 2.6. PDL- 1



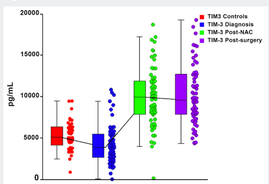
PD-L1 Comparisons	p-value
Control vs Pre-Treatment	0,00001
Control vs Post-Chemotherapy	0,00000
Control vs Post-Surgery	0,00174
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,86673

Figure 2.7. PD-1



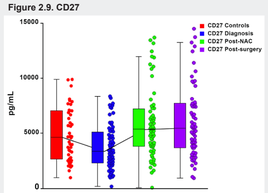
PD-1 Comparisons	p-value
Control vs Pre-Treatment	0,51577
Control vs Post-Chemotherapy	0,57531
Control vs Post-Surgery	0,64997
Pre-Treatment vs Post -Chemotherapy	0,78586
Pre-Treatment vs Post -Surgery	0,18600
Post Chemotherapy vs Post-Surgery	0,31017

Figure 2.8. TIM-3



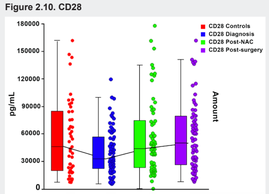
TIM-3 Comparisons	p-value
Control vs Pre-Treatment	0,00048
Control vs Post-Chemotherapy	0,00000
Control vs Post-Surgery	0,00000
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	1,00000

Figure 2.9. CD27



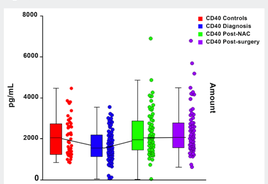
CD27 Comparisons	p-value
Control vs Pre-Treatment	0,02431
Control vs Post-Chemotherapy	0,11413
Control vs Post-Surgery	0,09949
Pre-Treatment vs Post -Chemotherapy	0,00001
Pre-Treatment vs Post -Surgery	0,00001
Post Chemotherapy vs Post-Surgery	0,81054

Figure 2.10. CD28



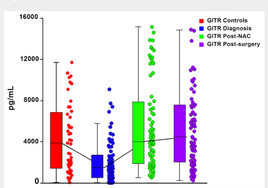
CD28 Comparisons	p-value
Control vs Pre-Treatment	0,12476
Control vs Post-Chemotherapy	0,80529
Control vs Post-Surgery	0,56390
Pre-Treatment vs Post -Chemotherapy	0,04156
Pre-Treatment vs Post -Surgery	0,00683
Post Chemotherapy vs Post-Surgery	0,57046

Figure 2.11. CD40



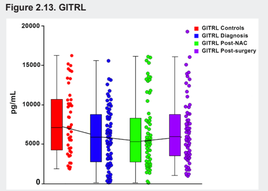
CD40 Comparisons	p-value
Control vs Pre-Treatment	0,00099
Control vs Post-Chemotherapy	0,48724
Control vs Post-Surgery	0,64997
Pre-Treatment vs Post -Chemotherapy	0,00029
Pre-Treatment vs Post -Surgery	0,00005
Post Chemotherapy vs Post-Surgery	0,62028

Figure 2.12. GITR



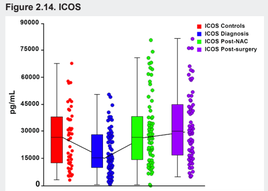
GITR Comparisons	p-value
Control vs Pre-Treatment	0,00008
Control vs Post-Chemotherapy	0,13616
Control vs Post-Surgery	0,15151
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,97769

Figure 2.13. GITRL



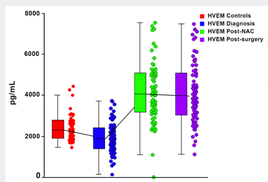
GITRL Comparisons	p-value
Control vs Pre-Treatment	0,01992
Control vs Post-Chemotherapy	0,05395
Control vs Post-Surgery	0,09613
Pre-Treatment vs Post -Chemotherapy	0,80435
Pre-Treatment vs Post -Surgery	0,36336
Post Chemotherapy vs Post-Surgery	0,52264

Figure 2.14. ICOS



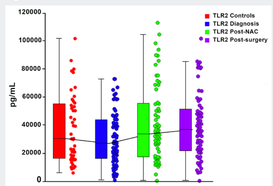
ICOS Comparisons	p-value
Control vs Pre-Treatment	0,00874
Control vs Post-Chemotherapy	0,38213
Control vs Post-Surgery	0,08243
Pre-Treatment vs Post -Chemotherapy	0,00016
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,37507

Figure 2.15. HVEM



HVEM Comparisons	p-value
Control vs Pre-Treatment	0,00041
Control vs Post-Chemotherapy	0,00000
Control vs Post-Surgery	0,22939
Pre-Treatment vs Post -Chemotherapy	0,00000
Pre-Treatment vs Post -Surgery	0,00000
Post Chemotherapy vs Post-Surgery	0,62594

Figure 2.16. TLR-2



TRL-2 Comparisons	p-value
Control vs Pre-Treatment	0,18058
Control vs Post-Chemotherapy	0,54513
Control vs Post-Surgery	0,49076
Pre-Treatment vs Post -Chemotherapy	0,02578
Pre-Treatment vs Post -Surgery	0,00940
Post Chemotherapy vs Post-Surgery	0,77818

Table 4. Comparison between the median pre-treatment ICM levels of the patients attaining a pCR and patients not attaining a pCR.

ICM	Pre-Treatment pCR (median pg/ml)	Pre-Treatmetn no pCR (median pg/ml)	p-value
BTLA	11158,79	20805,06	0,09381
CD80	1587,38	1758,04	0,37104
CD86	11140,02	12806,83	0,35118
CTLA-4	1567,38	1959,23	0,3447
LAG-3	123654,2	144059,1	0,33199
PD-L1	1625,73	1966,8	0,27858
PD-1	11086,85	13265,72	0,23135
TIM-3	3909,58	3422,375	0,90972
CD27	3150,51	3440,615	0,32575
CD28	31785,36	40785,61	0,24131
CD40	1440,05	1730,97	0,24132
GITR	1264,8	1566,92	0,46494
GITRL	5158,15	6925,995	0,09258
ICOS	14399,75	15777,33	0,31959
HVEM	1858,66	1802,72	0,60547
TLR-2	23846,32	30018,63	0,25684

There was no significant difference between the median pre-treatment ICM levels of the patients that attained a pCR compared to those patients that did not attain a pCR.

Conclusions

- **We identified low levels of stimulatory and inhibitory ICMs in newly diagnosed, non-metastatic breast cancer patients compared to healthy controls.**
- **Following treatment, with the exception of CTLA-4, most of these pre-treatment abnormalities of systemic ICM levels corrected.**
- **Neo-adjuvant chemotherapy is associated with upregulation of sPD-L1 and most other ICMs.**
- **These results indicate that early breast cancer is associated with down-regulation of soluble stimulatory and inhibitory ICMs.**
- **Newly diagnosed early breast cancer patients appear to have generalized immune-suppression independent of subtype and stage.**
- **To our knowledge, this is the first study to describe the effect of treatment on systemic ICMs in early breast cancer patients.**

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Corresponding author: bernardo.rapoport@up.ac.za