

¹Department of Otolaryngology, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, ²Department of Microbiology and Immunology, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, ³Department of Public Health Sciences and Sylvester Biostatistics Core Resource, University of Miami Miller School of Medicine, ⁴Department of Otolaryngology, University of California Davis Medical Center, ⁵Department of Pathology, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine

Introduction

Myeloid derived suppressor cells (MDSC) play a key role in tumor progression, in immune exclusion, and in preventing T cell infiltration in the tumor (Lu et al, 2016, Moschetta et al, 2017, Ameratunga et al, 2018, Chen et al, 2018, Parikh et al 2018a, Parikh et al 2018b, Sacadalan et al 2018, Kim et al 2019). We have previously shown in randomized placebo controlled clinical trials that PDE5 inhibition with Tadalafil can lower MDSC and regulatory T cells (Tregs) in the blood and at the tumor site, and significantly increased the anti-tumor T cell response evaluated by assessing the proliferation of magnetically purified CD3 T cells isolated before and after treatment to autologous dendritic cells pulsed with the autologous tumor. (Califano et al., 2015; Weed et al., 2015) These clinical trials did not demonstrate clinical benefit with Tadalafil administration alone, and a three arm randomized phase II clinical trial was designed with a combinatorial immunotherapy approach using Tadalafil and an anti-Mucin (MUC)1 vaccine with poly ICLC as adjuvant with non-randomized control arm of otherwise eligible patients undergoing surgical salvage alone but unwilling to receive study treatment (NCT02544880). This trial incorporated an open label single dose phase I lead in and was designed with planned safety analysis and interim immunologic analysis after 6 patients completed 2 courses of study treatment without treatment limiting toxicity (TLT) or had TLT up to the end of course 2. Eligible patients had recurrent or second primary stage III or IV squamous cell carcinoma (SCC) of the oral cavity, oropharynx, hypopharynx or larynx with surgically resectable disease and were recommended to have definitive salvage surgery, and must have had prior radiation therapy to the head and neck. The anti-MUC1 vaccine with poly ICLC adjuvant was chosen to prime an immune response against HNSCC as MUC1 has been shown as the immune dominant antigen for CD4 and CD8 T cells in 80% of patients with HNSCC (Beckhove et al, 2010). All patients on the phase I lead in were to receive one course of Tadalafil with anti MUC1/polyICLC vaccine pre-operatively with an additional 3 courses planned up to 7 months postoperatively and a final course of vaccine only at one year post operative, with all patients followed until 2 years post surgery. Safety was the primary endpoint of the Phase I trial, with secondary endpoints of immune response and recurrence-free survival (RFS). Accrual of non-randomized controls began during the phase I lead in.

Results of the interim safety and immunologic analyses of the phase I lead in trial have been previously published (Weed et al, 2019) and are summarized here. The combined treatment of Tadalafil and MUC1/polyICLC vaccine was well tolerated with no serious adverse events or treatment limiting toxicities (TLTs) as primary endpoint. Image cytometry analysis of scanned tumors in this interim analysis also indicated that the PDE5 inhibitor Tadalafil in conjunction with the MUC1/polyICLC vaccine effectively reduced the number of PDL1+macrophages present at the tumor edge, and increased the number of activated tumor infiltrating T cells, suggesting reversion of immune exclusion. However, this analysis also confirmed that CD163 negative cells within the tumor upregulated PDL1 after treatment, suggesting the instauration of additional mechanisms of immune evasion. These results prompted suspension of the planned phase II randomized trial of Tadalafil and MUC1/polyICLC vaccine with consideration of incorporating checkpoint inhibition with Tadalafil and MUC1/polyICLC vaccination in a redesigned future trial. The following is a report of RFS and overall survival of the Phase I lead in trial at the 2 year follow up point post surgical salvage in comparison with non-randomized controls undergoing salvage surgery only.

Methods

Patients were followed up to two years for recurrence and overall survival. RFS is defined as the time from date of surgery to the date of first documented recurrence. Patients who had a second primary prior to recurrence or who died from a cause not related to recurrence were censored at the time of documentation of those events. Patients alive or those lost to follow-up were censored at the last date of documented recurrence-free status, with follow up for recurrence free status continued until documented progression and/or death, or until 2 years post surgical salvage was reached. While not a defined secondary endpoint of the trial, overall survival (OS) was also analyzed as an exploratory endpoint, and defined as the elapsed time from date of surgery to date of death. Alive patients had follow up for OS censored at date of last known alive status or at two years post surgical salvage. Summary of patient's characteristics by arm were compared using the Fisher's exact test or the Student t-test. RFS and OS curves by arm were estimated using the Kaplan-Meier method and compared using the log rank test. Given the small number of events in our study, Cox regression analysis with Firth's bias correction method was employed to estimate hazard ratios and corresponding 95% confidence intervals and p values from univariable and multivariable models. The Firth method is a penalized likelihood approach to reducing small-sample bias in maximum likelihood estimation. Multivariable analysis was limited, fitting models including arm and adjustment for one or two additional variables. The analyses were performed in SAS 9.4.

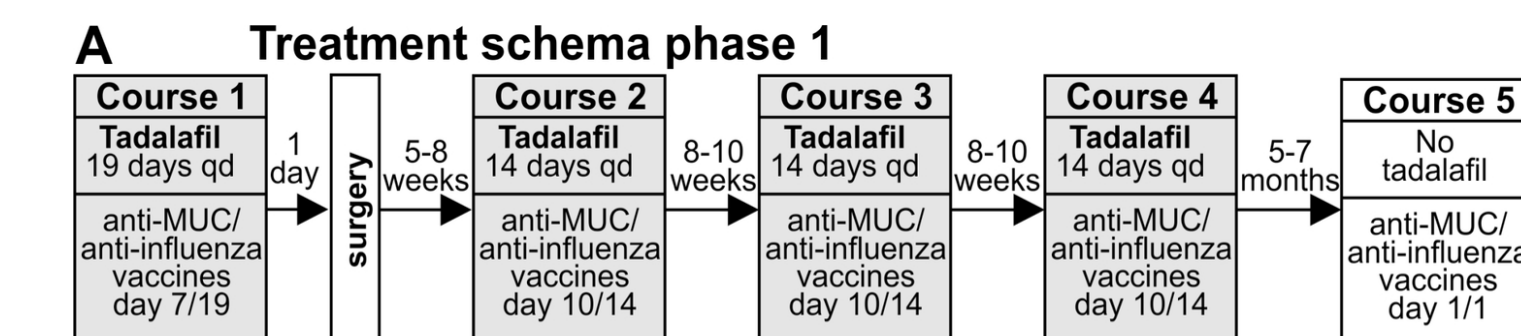
Results

A total of 15 patients were enrolled, 7 as controls and 8 to the active treatment arm. Study subjects were considered evaluable for phase I safety analysis once they completed Course 2 or if they experienced a TLT prior to completion of Course 2. The phase I lead in was designed to accrue 6 evaluable patients for safety analysis. One of 8 patients on the active treatment arm was non-compliant with study drug administration and was withdrawn at the beginning of course 1, and 1 of 8 patients developed disease progression following early tumor recurrence after lengthy recovery from surgery and was withdrawn from the study without receiving course 2 study treatment. An eighth patient was accrued to the treatment arm to yield 6 evaluable patients for TLT analysis. Any patient who received study treatment was evaluable for analysis of RFS, yielding 7 of 8 for RFS analysis. Safety analysis was planned following completion of Course 2 for the sixth evaluable study subject.

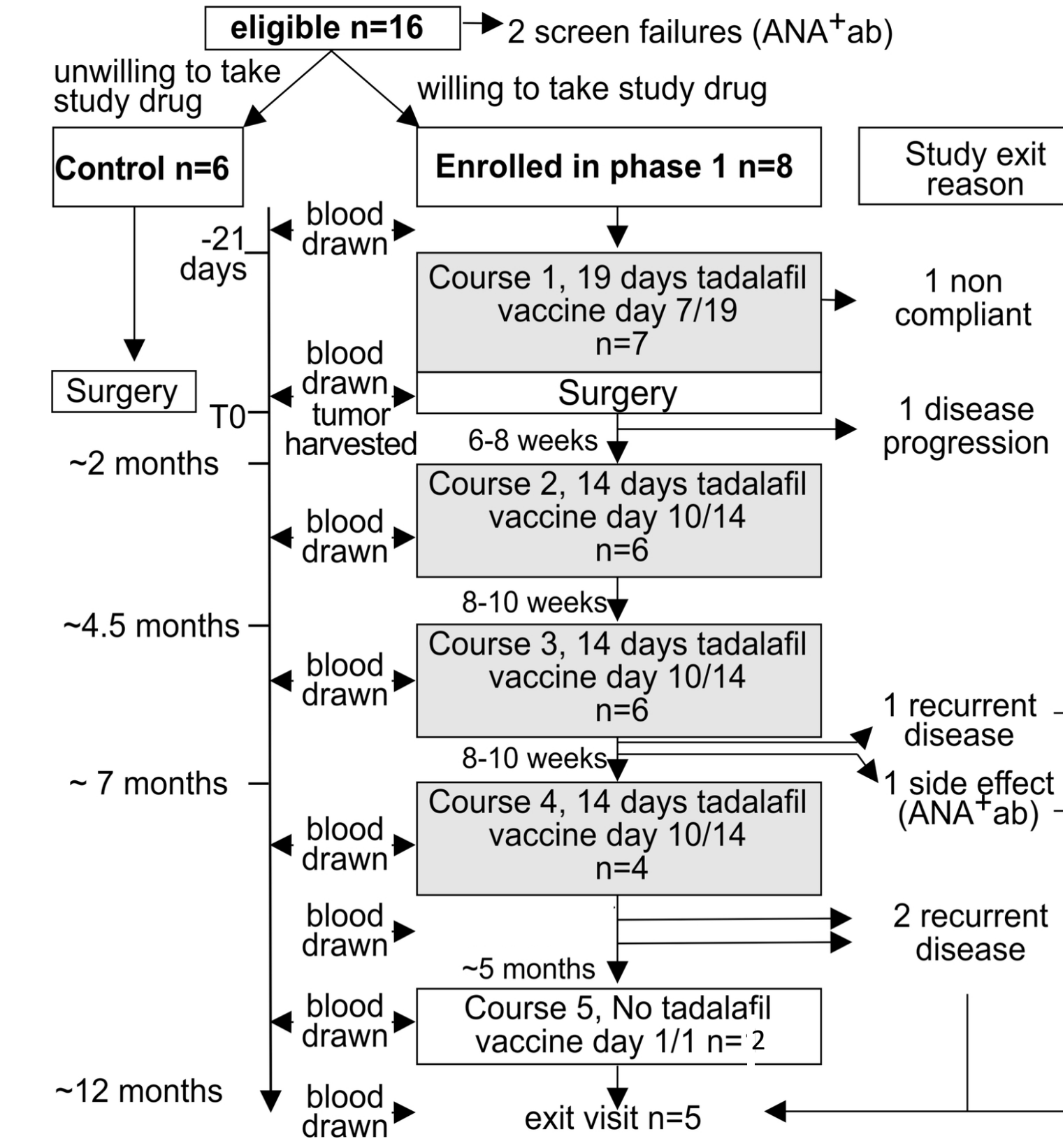
Clinicopathologic characteristics of treatment and control groups were similar. Disease sites for 7 evaluable treatment patients included oral cavity (n=2), oropharynx (n=3), and larynx (n=2), while disease sites of control patients included oral cavity (n=4), oropharynx (n=2), and larynx (n=1). Recurrent pathologic summary tumor summary stage (AJCC 7th edition) for treated and control patients was similar, with 6 stage IV and 1 stage III in the treatment arm, and 5 stage IV, 1 stage III and 1 stage II in the control arm (all patients were clinically judged to be stage III or IV at enrollment). Three of the 14 evaluable patients had p16 positive tumors consistent with HPV related malignancies, including 1 control and 2 treatment patients. All patients were previously irradiated as per study protocol, with 5 of 7 on both treatment and control arms receiving chemotherapy with radiation therapy as part of their prior treatment. Interim safety analysis has been previously reported with no evaluable patients experiencing TLT through completion of course 2. Follow-up analysis shows no evidence of TLTs through the remainder of completed courses in all evaluable patients.

Six of 7 patients in the treatment arm and 3 of 7 patients in the control arm had tumor recurrences within the first two years post-surgery. There was no statistically significant difference between arms on univariable analysis of recurrence-free survival (RFS) (treatment vs. control HR=1.84, 95% CI 0.46, 7.44; p=0.390). In multivariable analysis of RFS controlling for positive margin status and smoking history revealed an important influence of these additional variables on likelihood of recurrence, and no significant difference between treatment vs. control arms (HR=0.44, 95% CI 0.08, 2.36; p=0.334). In this multivariable model, there was evidence or worse RFS for patients with positive margins compared to negative margins (HR=25.18, 95% CI (2.10, 302.5; p=0.011), and for patients with smoking history versus without smoking history (HR= 8.48, 95% CI 1.04, 69.17; p=0.046). Four patients in each treatment arm were alive at the 2-year endpoint, 3 of 4 with disease in the treatment arm and 1 of 4 with disease in the control arm, with overall survival nearly equivalent between treated and control patients (treatment vs. control HR=0.93, 95% CI 0.19, 4.62; p=0.927). It should be noted that 1 of 6 recurrent patients in the treatment arm was included in the analysis as recurrent although was treated clinically as a second primary SCC of the lung with curative intent. Additional sites of distant metastatic disease subsequently developed post pneumonectomy and adjuvant chemotherapy in this patient. Analyzing this patient as recurrent was chosen given the similar histology and inability to definitively prove the lung lesion was second primary rather than first site of metastatic disease from the HN primary cancer.

Consort Flow Diagram



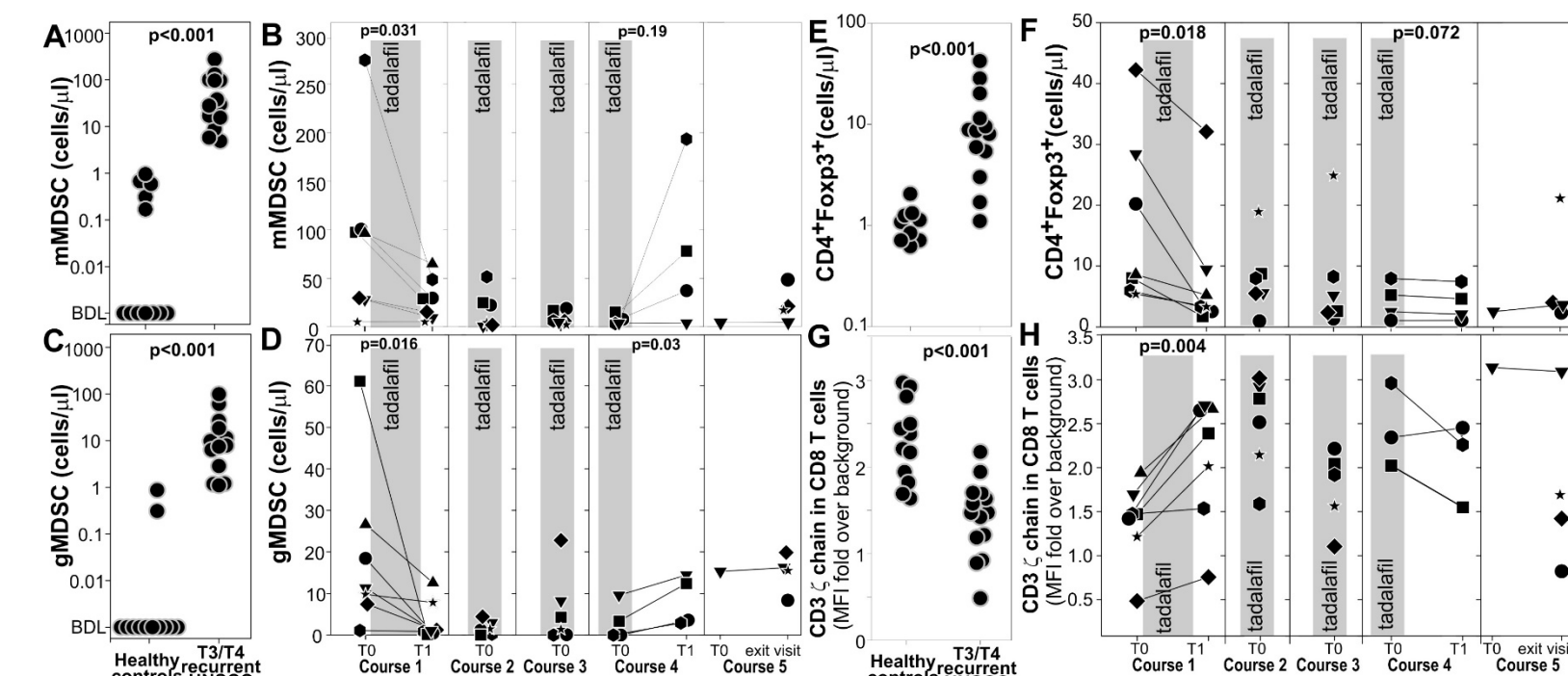
Clinical trial flow chart



Conflict of Interest, Funding, and Acknowledgements

Paolo Serafini is named as inventor in patent owned by Johns Hopkins University regarding the use of PDE5 inhibitors as immune modulator. Beside this potential future conflict, the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This work was possible by the support of Sylvester Cancer Center clinical trial accelerated grant and the Sylvester Cancer Center NIH Bridge award awarded to **DTW** and **PS**, and the Alpha Omega Alpha Postgraduate Award awarded to **MA**. We would like to thank Dr. O. Finn (University of Pittsburgh) and her laboratory for providing the MUC1 vaccine and protocols for IHC and ELISA, and Dr. Salazar (Oncovir) for providing polyCLC (hiltono) at production cost.

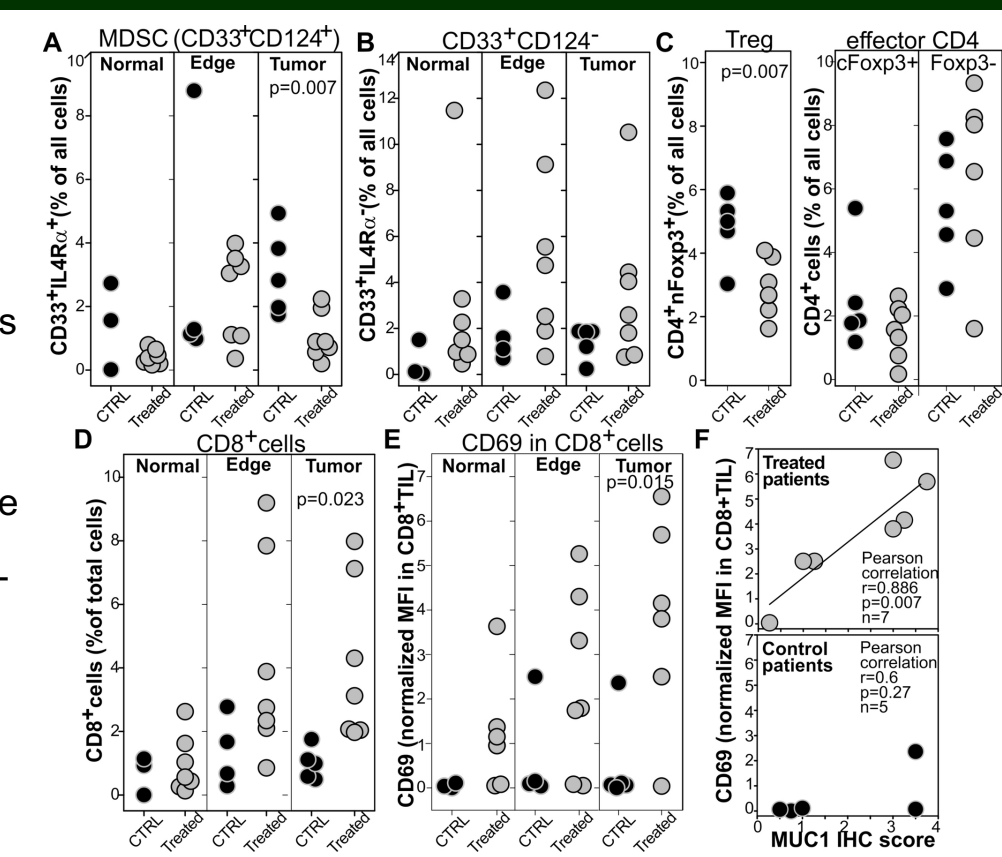
Tadalafil and MUC1 vaccine lowers circulating MDSCs and Treg and restores the expression of CD3 ζ -chain in CD8 T cells



mMDSC, gMDSC, Treg, and the expression of CD3 ζ -chain expression on CD8 T cells was evaluated by multicolor flow cytometry on fresh blood of the enrolled patients or on age matched healthy donors (A, C, E, G). Leukocyte subsets were enumerated with "123 beads" Two ways T test value are reported. The same subsets were evaluated longitudinally in the patients enrolled in the treatment arm. The gray area correspond to the Tadalafil treatment (B, D, F, H).

Tumors from patients treated with Tadalafil and MUC1/polyICLC vaccine show a lower infiltration of MDSCs and Treg and a higher infiltration of activated CD8 in the tumor bed

Computer based image cytometry was performed to enumerate the number of **A)** MDC5, **B)** IL4R α -myeloid cells, **C)** CD4 $^{+}$ T cell subsets, or **D)** CD8 $^{+}$ T cells. **E)** CD69 expression within the CD8 $^{+}$ cells reported normalized on the CD69 expression on all the cells evaluated. Depending on the region of interest evaluated, at least 105-106 cells were analyzed. **F)** The expression of CD69 in CD8 $^{+}$ T cells was plotted against MUC1 IHC score of the corresponding tumor. Two ways T test and relevant pearson correlation parameters are reported.

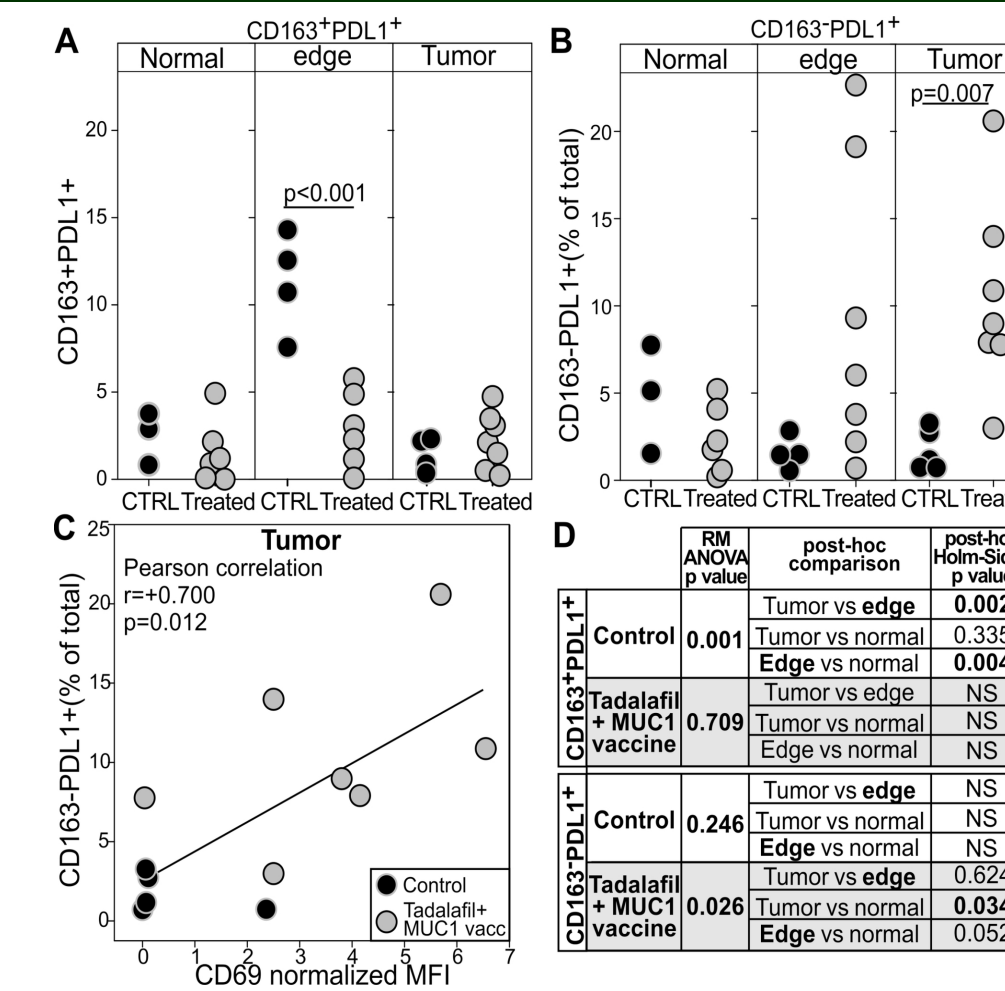


Tadalafil and MUC1 vaccine treatments modulate the expression of PDL1 in the tumor microenvironment

The expression of PDL1 within the CD163+ (A) or the CD163- cells (B) was quantified by image cytometry in the tumor, at the tumor edge, or in "normal" tissue surrounding the tumor in the tumor specimen from the control (black filled circle) or Tadalafal and MUC1/polyICLC vaccine treated (gray filled circle) patients. Two way t test p values are reported.

C Correlation between the expression of CD69 in the CD8+ T cells and PDL1 expression on the CD163-cells.

D) Summary of the one way RM ANOVA analysis.



Impact of positive margins and smoking history on RFS

Cox Proportional Hazards Regression Analysis for RFS and OS

		RFS (9 events in 14 patients)		OS (6 events in 14 patients)	
Variables	Categories	HR (95% CI)	P	HR (95% CI)	P
Univariable models					
Arm	T vs C (Ref.)	1.84 (0.46, 7.44)	0.390	0.93 (0.19, 4.62)	0.927
Smoking History	Yes vs. No (Ref.)	0.50 (0.71, 28.68)	0.111	7.35 (0.32, 166.9)	0.211
Smoking status	Quit=>5y vs. Never Smoker (Ref.)	5.00 (0.69, 36.54)	0.112	11.30 (0.43, 299.2)	0.147
	Smoker/Quit=>5y vs. Never Smoker (Ref.)	4.45 (0.56, 35.55)	0.159	4.00 (0.11, 147.0)	0.451
HPV2	Pos. vs. Neg./NA (Ref.)	0.64 (0.09, 23.58)	0.064	2.81 (0.52, 15.34)	0.232
Trial Tumor Site	OC vs. Larynx (Ref.)	4.50 (0.91, 29.55)	0.446	1.02 (0.11, 9.86)	0.985
	Oro vs Larynx (Ref.)	1.48 (0.31, 6.95)	0.620	1.17 (0.25, 18.83)	0.453
gTNM	4 vs. <4 (Ref.)	1.12 (0.28, 4.44)	0.869	1.88 (0.27, 13.27)	0.528
pTNM	4 vs. <4 (Ref.)	0.72 (0.16, 3.24)	0.656	0.40 (0.07, 2.17)	0.290
Positive Margins	Yes vs. No (Ref.)	12.21 (1.66, 90.50)	0.014	2.24 (0.45, 11.23)	0.326
Trial LVI	Yes vs. No (Ref.)	4.91 (1.00, 24.00)	0.049	1.22 (0.25, 6.05)	0.869
Trial PNI	Yes vs. No (Ref.)	4.24 (0.67, 27.07)	0.126	0.97 (0.18, 5.15)	0.968
Prior Chemo	Yes vs. No (Ref.)	0.66 (0.17, 2.63)	0.556	1.88 (0.27, 13.27)	0.528
Age, in years	>= 65 vs. <65	1.34 (0.34, 5.30)	0.680	1.52 (0.28, 8.18)	0.623
Age, in years	1 year increase	0.97 (0.87, 1.08)	0.583	1.04 (0.91, 1.18)	0.585
Multivariable model 1					
Arm	T vs C (Ref.)	0.99 (0.19, 5.07)		0.987	
Positive Margins	Yes vs. No (Ref.)	11.54 (1.41, 94.72)		0.023	
Multivariable model 2					
Arm	T vs C (Ref.)	0.44 (0.08, 2.36)		0.334	
Positive Margins	Yes vs. No (Ref.)	25.18 (2.10, 302.5)		0.011	
Smoking History	Yes vs. No (Ref.)	8.48 (1.04, 69.17)		0.046	

HR: hazard ratio, 95%CI: 95% confidence interval. P: p value, <p<0.10 in bold.

Discussion & Conclusions

While the Phase I lead in was not designed to assess RFS as primary endpoint given the limited number of patients enrolled, some insights can be gained from this small sample size of treated and matched non-randomized controls in analysis of 2-year follow-up for RFS and OS in comparison with the secondary endpoint of immune response for this group. This study confirms the high risk of recurrence in stage III or IV previously irradiated patients undergoing salvage surgery for HNSCC. As previously reported in the trial's interim analysis, underglycosylated MUC1 as a tumor associated antigen is widely expressed in these patients with recurrent HNSCC, and the combined treatment of Tadalafil and anti-MUC1/poly/ICLC vaccine was well tolerated. The combined treatment did show evidence of reversion of immune exclusion by lowering MDSCs and Treg at the tumor site and in peripheral blood and an increase in the number of activated tumor infiltrating T cells, however study treatment also resulted in CD163 negative cells within the tumor upregulating PDL1 expression, suggesting the instauration of additional mechanisms of immune evasion. This important finding prompted suspension of the Phase II component of the trial, and is likely a contributing cause of the finding that RFS and OS of the small sample size of patients evaluated show no significant difference between treated and control groups. While the number of recurrences is greater in the treated arm, raising the question of possible increased risk of recurrence due to study treatment albeit not statistically significant, multivariable analysis of RFS controlling for positive margins and smoking status shows an important influence of these additional variables on likelihood of recurrence between treatment vs. control arms. This study provides rationale for consideration of combinatorial immunotherapy with checkpoint inhibition, Tadalafil administration, and anti-MUC1/poly/ICLC vaccination in this challenging patient population.

References

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