Evaluating Persistent Abnormal Neuromuscular Junction Function Following Chronic Volumetric Muscle Loss in Middle-Aged Mice

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INTRODUCTION: Volumetric muscle loss (VML) – i.e., loss of a critical volume (>20%) of a muscle – contributes to 50% of injury-related retirement of military forces[1]. These injuries result in a disproportionate loss in contractile function that hinders meaningful recovery even with physiotherapy. Current clinical and experimental treatments for VML fill the lost volume but do not result in functional recovery, even when skeletal muscle tissue appears grossly normal. In young, highly regenerative mice, previous attempts at treating VML result in complete muscle regeneration but suboptimal function[2]. Despite this gap in histological and functional recovery, VML research is lacking in two main areas: utilization of a clinically representative model of VML injury and treatment, and investigation of the functional deficits in remaining and regenerated skeletal muscle. The majority of VML models create and treat borderline critical acute injuries in young mice[3], which are highly regenerative and not reflective of the age of patients or the chronicity of injury at presentation for treatment. We hypothesize that functional deficits exist in chronic VML due to abnormal patterning and function at the neuromuscular junction bands, where muscles receive signals from nerves. Herein, we characterize a ***chronic and significant (50%) VML injury*** in ***middle-aged mice*** using quantitative lightsheet microscopy (QLSM) to efficiently and wholistically analyze NMJs, conventional 2D histology, functional testing, and combine this data with single-nucleus transcriptomics analysis to understand the molecular mechanism of functional deficiencies in skeletal muscle after VML.

METHODS: Function and Histology. All animal experiments received IACUC approval. Male (n=41) and female (n=36) mice aged 9-12 months had 21-25 mg (~50% volume) of their tibialis anterior resected from the center midline. After a month, the contractility of the TA was assessed every 2 weeks by in vivo functional testing. Animals were followed for 16 weeks and harvested at either week 8 or 16 for QLSM or conventional histology. Animals expressing tdTomato under the control of BAF53b protein, a pan-neural structural protein, - BAF53b::tdT were used for QLSM images. BAF53b::tdT mice(n=32) were injected with fluorescent alpha-bungarotoxin to visualize acetylcholine receptors (**Fig. 1 A**). NMJ quantity, innervation, and gross patterning were analyzed using QLSM, and detailed structures were semi-quantitatively assessed using confocal images. Muscle cross-section (CSA), myofiber CSA, vascularization, and macrophage infiltration were assessed using Masson's Trichrome and immunofluorescent imaging of 10µm transverse cryosections in the mid-defect region. **Statistics:** Repeated measures ANOVA was used to analyze functional data and body mass, while two-way or one-way ANOVA was used for multiple or single timepoint assessments. **Transcriptomics.** TA muscles from male animals (n=6 per group), received VML or sham surgery at 1 week following surgery. Nuclei were isolated from flash frozen muscle and prepared using the 10X Genomics platform to target 20,0000 nuclei at a sequencing depth of 30,000 reads. Data were analyzed following quality control (QC) to remove low-quality nuclei. Analysis will identify differentially expressed genes, ligand receptor interactions, and gene regulatory pathways between conditions.

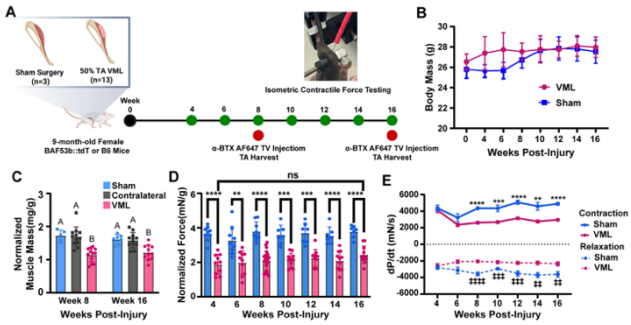

Figure 1. Chronic VML in Middle-Aged mice causes persistent functional deficit. **A**. Study Outline **B**. Stable mouse mass over 16 weeks, with persistent mass (**C**). functional deficit (**D**) and contraction and relation rates (**E**).

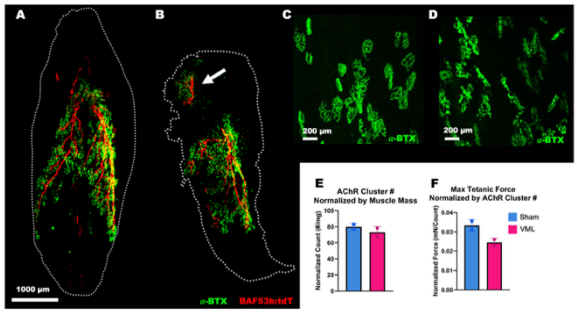

Figure 2. Robust NMJ Analysis in optically cleared TA muscle **A**. Endogenous tdTomato (red) highlights normal nerve morphology, and injected aBTX(green) shows NMJ patterning **B**. VML causes NMJ loss and ectopic nerve patterning(arrow). Confocal imaging shows normal NMJ morphology (**C**) compared to VML at 16 weeks (**D**). Mass appropriate AChRs are maintained (**E**), but less force is produced by clusters(**F**).

RESULTS: Our data demonstrated that VML had no long-term impact on mouse growth (**Fig. 1 B**) but resulted in chronic muscle mass and functional deficit (**Fig. 1 C, D**), in both sexes, which plateaus between 4 and 8 weeks following VML. Beyond the limited maximum tetanic force, a significant reduction in rates of contraction and relaxation was also observed (**Fig. 1 E**). Current data points to VML causing a reduction in the quantity of NMJs, and interestingly, nerve and NMJ ectopy. (**Fig. 2 A ,B, E**), and increased AChR fragmentation (**Fig. 2 C, D**). When used to normalize the functional data, preliminary data (n=2) indicate that NMJs are less functional in VML compared to aged and sex matched shams (**Fig. 2 F**). Though treatment of VML with engineered grafts resulted in recovery of skeletal muscle tissue and NMJ number, a similar function pattern was observed (Data not shown). Following initial QC of nuclei, 57,464 were analyzed following batch correction and identification of highly variable genes. Clustering and analysis identified major nuclei populations (**Fig. 3**). Next steps will identify DEGs between conditions across cell types and differences in inter- and intra-cellular communication networks.

DISCUSSION: Previous work by our group has demonstrated that younger mice recover significant muscle mass when treated and modest improvement without treatment, but no functional recovery[2]. Here, we demonstrated persistent functional deficit from as early as 4 weeks post-injury, which contrasts with a chronic injury in 3-month-old C57BL/6 mice showing continued functional improvement up to 16 weeks post-injury[3]. QLSM imaging facilitated gross, quantitative changes regarding aberrant patterns of innervation and NMJ clustering. In combination with transcriptomic changes, it will provide unique insight into neuromuscular changes following VML injury. Ongoing analysis will focus on identifying abnormal transcription patterns within muscle, especially with NMJ-associated nuclei.

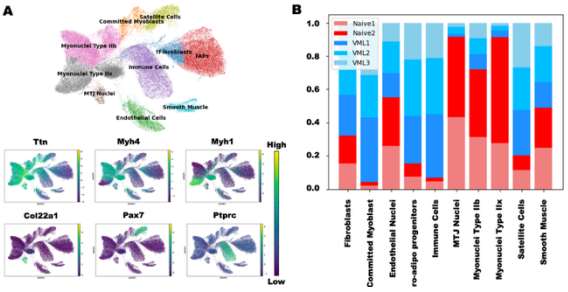

Figure 3. Single nuclei RNA sequencing enables identification nuclei populations **A**. UMAPs enable visualization of major nuclei populations and mean expression of some canonical marker genes **B**. VML results in a relative increase in satellite cell expansion and myoblast committal and significant FAP and immune cell expansion.

SIGNIFICANCE: This study characterizes a model of VML consistent with clinical presentation. Additionally, transcriptomic data will help to elucidate the mechanisms that drive functional deficits in VML recovery and toward identifying precise therapeutic targets.

RELEVANCE: VML is a common musculoskeletal source of morbidity among military and civilian populations. Large limb muscles modelled here will be a translational challenge but provide a mechanistic understanding for the treatment of critical muscles of the head and neck.

REFERENCES: [1] Corona, B. T et al. (2015). J. Rehabil. Res. Dev, 52(7). [2] Mihaly, E et al. (2025). Adv. Healthc. Mater., 14(2), 2403028. [3] Hoffman, D. B. et al. (2024). *Exp. Neuro*, *382*, 114996.