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Transcriptomic characterisation of recurrent exertional rhabdomyolysis using bulk and single nuclei RNA Seq in muscle and blood of Thoroughbred horses

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Introduction

Recurrent exertional rhabdomyolysis (RER) poses both welfare and economic problems, it is a painful myopathy and an episode results in the horse being out of work for an average of 7 days (Isgren *et al*., 2010). Current treatments focus on management of clinical signs, however, the underlying causes remain poorly understood, limiting the ability to prevent RER or reduce its prevalence. Current diagnosis is dependent on observations of clinical signs, blood biochemistry for raised AST and CK levels and histopathological examination of, usually, the semimembranosus muscle, this is, however, an invasive procedure (Rivero and Piercy, 2008). For this reason, better understanding of the condition, including identification of biomarkers present in blood would be greatly beneficial. A genomic link has been identified, h2=0.39-0.49 (Lindsay-McGee *et al*., 2024) as mentioned above, therefore, identification of horses at higher risk of RER and earlier intervention by altered management and training strategies would help to assist in reducing incidences. Further, using genomic biomarkers for risk, future breeding strategies could be influenced to reduce prevalence in the population. Here we have generated bulk RNA-Seq in the semimembranosus (SM) muscle tissue that is usually affected to study the local transcriptomic signature as well as in blood to study the systematic signature of RER and identify potential biomarkers for RER susceptibility that could reduce reliance on the current invasive biopsy for clinical diagnosis. We have also generated single nuclei(sn)RNA-Seq data in SM to gain better biological insights into the disease at a fibre type (cellular) level.

Materials & Methods

Ethical approval was provided for this study by the RVC Clinical Research Ethical Review Board (CRERB). Semimembranosus muscle (SM) sample biopsies were collected with informed owner consent by licensed veterinarians and sent to the Comparative Neuromuscular Diseases Laboratory at the Royal Veterinary College for diagnostic purposes. Briefly, horses (8 cases and 13 controls) were sedated and the biopsy site was aseptically prepared. Local anaesthetic was administered subcutaneously and a 3×3 cm sample obtained by two parallel 3 cm incisions and undermining an 8 mm-deep strip. The specimen was divided into ~10mm segments and flash-frozen in liquid nitrogen. For blood sample collection, residual blood (~3mL) from blood collected for clinical diagnostics and/or routine biochemistry by jugular vein puncture, was collected into Tempus™ Blood RNA Tubes (Invitrogen, USA) and RNA extracted using the Tempus™ Spin RNA Isolation Kit (Invitrogen, USA) following manufacturer's instructions.

For Bulk RNA-Seq, RNA was extracted from cryopreserved muscle biopsies using a trizol-chloroform protocol combined with the RNeasy commercial kit (Qiagen, Hilden, Germany). Briefly, ~30mg of muscle tissue was mechanically homogenised by Fastprep96 (MP Biomedicals', California, USA) in 1mL trizol with a steel ball and three rounds of bead beating

at 1200rpm, 30 seconds. Chloroform was added (300µL) and the sample vortexed and centrifuged (12,000rpm, 4ºC, 15minutes (Haier Biomedical, UK)). The upper clear aqueous phase was aspirated and isopropanol (500µL) added for a 10minute RT incubation before application to RNeasy spin columns and the extraction performed according to manufacturer's instructions. Bulk RNA-Seq library preparation and sequencing was outsourced to Novogene Ltd. (Cabridge, UK) using NovaSeq X Plus Series (PE150), 15Gb. Raw reads were quality checked and trimmed usingFastQC, multiQC and Trimmomatic (Andrews, 2010, Bolger *et al*., 2014, Ewels *et al*., 2016), respectively. Reads were pseudoaligned to the EquCab3.0 genome by Kallisto (Ensembl, 2026, Bray *et al*., 2016) and differentially expressed genes (DEGs) identified using DESeq2 and adjusted for effect size using apeglm (Zhu *et al*., 2019, Love *et al*., 2014). Functional enrichment analyses were performed using ClusterProfiler and PANTHER Overrepresentation test and summarised using REVIGO (Supek *et al*., 2011, Yu *et al*., 2012, Mi *et al*., 2013, Thomas *et al*., 2022).

For snRNA-Seq (5 cases and 7 controls), nuclei were extracted from cryopreserved muscle using the 10X Genomics (California, USA) Chromium Nuclei Isolation Kit with RNase Inhibitor Chromium™. 10X GEMs were generated using the Next GEM Single Cell 3' Kit v3.1 and GEM libraries sequenced by the Centre for Genomic Research (Liverpool, UK) using NovaSeq X Plus (PE150), cell recovery target of 10,000/sample. Cell counts were filtered using CellRanger and analyses conducted in Seurat in R (Hao *et al*., 2024, RCoreTeam, 2016). QC was performed per-sample, bespoke RNA thresholds were applied to remove low-complexity nuclei and multiplets. Filtered data were normalised, variable features identified and samples were integrated using CCA-based integration before computation of a shared nearest-neighbour graph and clustering (resolution=0.5). Cluster-specific marker genes were identified (min.pct = 0.25, log2FC threshold = 0.25) and cell type annotation applied using a data and knowledge driven approaches, (manual curation of Seurat designated markers combined with literature mining of equine orthologs to published human and mouse snRNA-Seq markers). DEGs per cell type were identified using DESeq2.

Results & Discussion

In blood we identified 91 DEGs at padj ≤0.05, 34 at log2fold change (LFC) ≤-1 or ≥1. Enrichment analysis found statistically significant (FDR≤0.05) >10 fold enrichment for retrotransposition and transposition. Bulk RNA-Seq in muscle showed separation of RER cases and controls by principal component analysis (PCA)(Fig. 1A) and identified 988 DEGs (padj ≤0.05, LFC ≤-1 or ≥1). Enrichment analysis found 31 statistically significantly enriched GO BPs, summarised by REVIGO (Figure 1B). These included cell communication, metabolism, lipid transport and metabolism and glutamate receptor clustering. SnRNA-Seq analysis identified 10 cell clusters (Fig.1C). The number of identified DEGs by cell type (padj ≤0.05) ranged from 15 (Immune cells (monocyte/macrophage like)) to 479 (Oxidative muscle fibres) (Fig.1D). Overlap of DEGs between cell types (Fig.1E) found the greatest overlap between endothelial and smooth muscle cells and with fibroblasts, with enrichment in the same direction. Overlapping DEGs (n=34) between snRNA-Seq and bulk muscle RNA-Seq(Fig.1F) were involved in actin filament polymerization and DNA recombination. Between snRNA-Seq and bulk blood RNA-Seq overlapping DEGs were involved in positive regulation of apoptotic process and intracellular signal transduction. Between bulk RNA-Seq of muscle and blood, the single overlapping gene was Ret Finger Protein Like 4B, predicted to enable ubiquitin protein ligase activity and involved in the innate immune response and gene expression regulation (NCBI, 2025).

Enrichment analyses of the snRNA-Seq DEGs found in healthy muscle the T-cell cluster enriched for RNA splicing and processing, macrophages enriched for purine catabolic and

metabolic process' and monocytes enriched for calcium ion transport and muscle contraction regulation. These suggest macrophages are more metabolically active in healthy tissue, supporting the tissue in clearance, remodelling and regeneration. Monocytes were also more supportive of Ca^{2+} signalling and normal excitation and contraction. Fibroblasts, stromal and connective, were enriched in healthy tissue similarly to the macrophage cluster; for purine catabolism and metabolism and for lipid transport and localisation. Finally, oxidative fibres in healthy tissue were enriched for purine catabolism and metabolism, lipid transport and localisation and extracellular structure and extracellular matrix organisation.

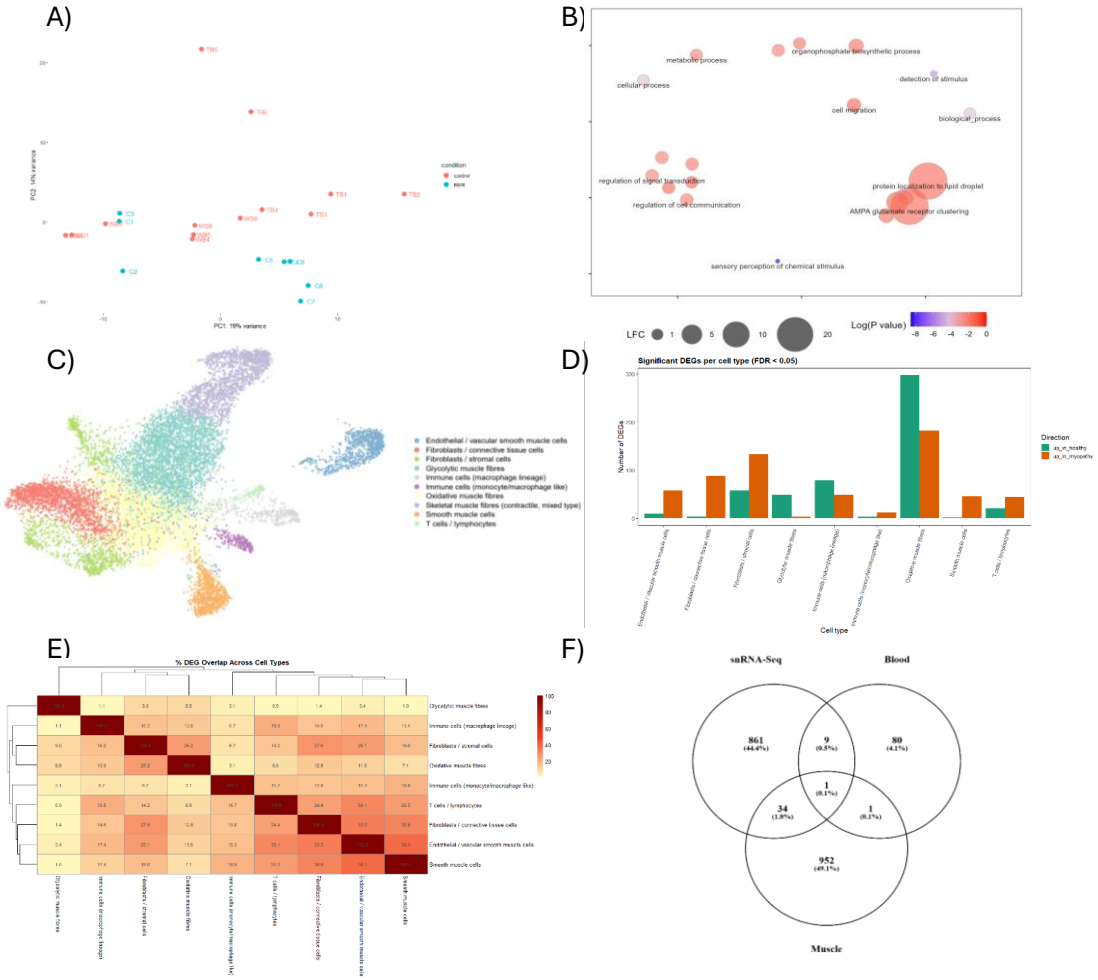

Figure 1. A) PCA plot of sample transcriptional profiles in SM bulk RNA-Seq (cases blue, controls red.) B) REVIGO plot of significantly enriched GO biological processes from identified DEGs (padj ≤0.05, and LFC ≤-1 or ≥1). C) UMAP of snRNA-Seq nuclei transcriptomic profiles, clusters coloured by cell type. D) Number of DEGs per cell type coloured by direction of enrichment. E) Overlap matrix of percentage overlapping DEGs across cell types. F) Venn diagram of overlapping DEGs between analyses (blood RNA-Seq p≤0.05, muscle RNA-Seq and snRNA-Seq p≤0.05 and LFC ≤-1 or ≥1).

Discussion

Downregulation of lipid transport and localisation pathways in RER muscle suggests reduced availability and use of fatty acids as an energy source, leading the tissue to become more dependent on glycogen for ATP production, thereby limiting metabolic flexibility and reducing mitochondrial efficiency. This increases; susceptibility to metabolic stress, production of reactive oxygen species (ROS) and cellular damage (Watt and Hoy, 2012). Previous studies of equine athletes found high intensity training resulted in a shift of muscle fibre types in skeletal

muscle from glycolytic to more oxidative, indicating a more lipid-dependant form of ATP biogenesis and reduced reliance on glycogen; a more metabolically flexible tissue with increased resilience to exercise (Kitaoka *et al*., 2012). Our findings reveal healthy muscle tissue displays metabolic flexibility and a supported and resilient environment. Downregulation of purine metabolism in our RER cases indicates dysregulation of AMP disposal and ATP regeneration contributing to energetic stress, this been observed to result in exercise intolerance (Operti *et al*., 1997, Zieliński *et al*., 2019). Immune, stromal, and myofibre populations all contribute efficient purine turnover and lipid handling, effective calcium regulation, and active extracellular maintenance. These support and facilitate efficient ATP homeostasis, mitochondrial function, and resistance to exercise-induced stress. Chronic immune activation, fibroblasts and stromal metabolic dysfunction and homeostasis, oxidative fibres metabolic and structural dysregulation all indicate reduced capacity for endurance and resistance to fatigue and stress. This correlates with enriched transposable elements pathways in blood, previously associated with exercise tolerance (Capomaccio *et al*., 2010, Capomaccio *et al*., 2013). In summary, the convergence of impairments observed here in muscle tissue from horses with RER, suggests metabolically inflexibility, Ca^{2+} dysregulation, oxidative stress, vulnerability to fatigue and overall reduced adaptability to the energetic and mechanical stresses of exercise.

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