

snRNA Transcriptomic Analysis to Assess the Role of AQP1 and AQP4 as Biomarkers for Duchenne Muscular Dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a severe X-linked genetic disorder affecting approximately 1 in 3,500 male births. Characterized by progressive muscle weakening and reduced life expectancy, DMD presents a significant challenge in identifying reliable genetic expression biomarkers due to its wide phenotypic variability. The potential roles of aquaporin 1 (AQP1) and aquaporin 4 (AQP4) as disease markers have been debated for over two decades. This study employs a novel single-nucleus RNA (snRNA) transcriptomic analysis to evaluate the expression of AQP1 and AQP4 in DMD muscle tissue. The results demonstrate a significant upregulation of AQP1 in DMD samples compared to controls, while AQP4 expression was elevated but did not reach statistical significance. These findings contribute to the understanding of aquaporin involvement in DMD pathophysiology and highlight the potential of AQP1 as a biomarker for the disease. Furthermore, this research addresses a critical gap in knowledge regarding the role of aquaporins in DMD and opens new avenues for future investigations into disease mechanisms and potential therapeutic targets.

1 Introduction

Duchenne muscular dystrophy is an X-linked recessive disorder characterized by its severe pathogenesis, progressively wasting the muscles of those suffering from the disease.

The earliest symptoms appear around 2–3 years of age, and the neuromuscular disease continues to destroy striated muscle cells throughout the patients' lives. Thus, in their early teens, most patients become wheelchair-dependent and require assisted ventilation in their twenties. Ultimately, their life expectancy is shortened—even with optimal care—to around 20 to 40 years old due to cardio-respiratory complications (Mercuri et al., 2019). However, modern advances in patient care have produced a significant increase in the median age amongst those patients born after 1990, to 28.1 years (95% CI 25.1, 30.3) (Broomfield et al., 2021).

As mentioned before, DMD is a genetic disease caused by a wide range of mutations in the dystrophin gene (Xp21). These mutations include deletions (60–70% of cases), duplications (5–15%), and point mutations, small deletions, or insertions (20%) (Duan et al., 2021).

These mutations affecting dystrphic, dystrophinopathies, result in a loss of myofiber integrity with repeated cycles of necrosis and regeneration (Venugopal & Pavlakis, 2024).

Despite numbers varying among sources, DMD is the most common neuromuscular disorder among children (Dowling et al., 2018). Due to it being an X-linked disorder, epidemiologically, males are more likely to be affected by Duchenne muscular dystrophy (~0.027% – 0.061% of male live-born infants) as opposed to 1 in 50 million live female births (Duan et al., 2021; Ryder et al., 2017). However, there is a lack of data, and I have found the original sources reporting these numbers to date back even two decades (Crisafulli et al., 2020). Therefore, the scope at which we are trying to evaluate the number of people affected might be quite restrained.

1.1 Clinical Features & Differential Muscle Involvement

As mentioned earlier, Duchenne muscular dystrophy is characterized by progressive muscle weakness. However, such weakness follows a distinct pattern of involvement (Nieves-Rodriguez et al., 2023). The proximal muscles of the lower limbs are usually affected first, leading to difficulties in running, climbing stairs, and rising from the floor. This proximal-to-distal progression is a hallmark of DMD, with the quadriceps and hip extensors being among the earliest and most severely affected muscle groups (Fornander et al., 2021). As shown in Figure 1, an increase in adipose tissue is characteristic of the disease.

On a different note, as the disease progresses, upper limb involvement becomes more pronounced, particularly in non-ambulant patients. Mazzone et al. (2011) developed a novel approach to assess the latter in SMA boys, highlighting the importance of monitoring distal muscle function—which in SMA's case, are also affected on later stages of the disease. Yet, their work underscores the need for comprehensive functional assessments throughout the disease course while accounting for differential muscle involvement (Mazzone et al., 2011).

Recent transcriptomic analysis by Nieves-Rodriguez et al. (2023) compared gene expression profiles between the vastus lateralis (VL) and tibialis anterior (TA) muscles in healthy individuals to understand potential factors influencing differential muscle susceptibility in DMD. They identified 3,410 differentially

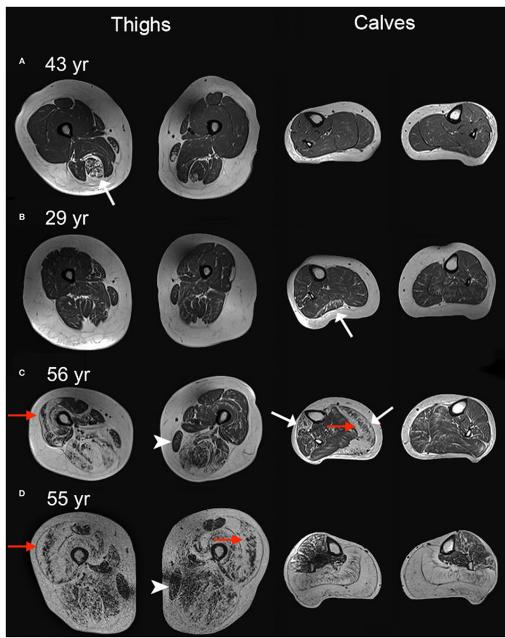


Figure 1: Four T1-weighted examples. T1-weighted MRI images of thighs and calves of four women heterozygote for pathogenic DMD variants. White represents fat tissue. (A–D) shows a range of muscle affection from mild to severe. Cases (C,D) are particularly affected with prominent replacement of muscle by fat. Correspondingly, muscle strength was compromised in these patients. From “Quantitative Muscle MRI and Clinical Findings in Women With Pathogenic Dystrophin Gene Variants,” by F. Fornander et al., 2021, *Frontiers in Neurology*, 12, p. 9. Copyright 2021 by the authors. Licensed under CC BY 4.0.

expressed genes between these muscle groups, including those involved in calcium signaling, extracellular matrix composition, and apoptotic regulation. This differential gene expression may partly explain why proximal muscles like the VL are typically affected earlier and more severely than distal muscles like the TA in DMD patients.

Understanding these pathogenic patterns is a much-needed step in order to develop better clinical treatments. However, this article focuses on the role of AQP4 and the data used is limited to VL muscle. Therefore, it is noteworthy that the results of this project do not illustrate the role that AQP4 might play in other muscles.

1.2 AQP1 and AQP4 as Potential Biomarkers

Aquaporin-4 (AQP4) plays a crucial role in maintaining water homeostasis, a process essential for osmotic balance and overall muscle function. Although simple diffusion allows for water movement across mammalian cell membranes, this mechanism alone is too slow to meet the rapid demands of cellular homeostasis. AQP4, along with other aquaporins, facilitates the swift water flux necessary for proper cellular function.

The importance of AQP4 is highlighted by its sig-

nificant localization in the neuromuscular system, particularly in the brain and astrocytes (Benfenati et al., 2011). While AQP4 plays a critical role in the brain, it is also a major regulatory factor in water transport within skeletal muscle, where it is expressed in the plasma membrane of myofibers (Frigeri et al., 1998). AQP4 exists in multiple isoforms, including the shorter AQP4-M23 and the longer AQP4-M1, as well as a newly identified splice variant, AQP4 Δ4, which may play a regulatory role in AQP4 expression and activity (De Bellis et al., 2014; Rossi et al., 2010).

In the context of Duchenne muscular dystrophy (DMD), AQP4 expression patterns have been studied in relation to $\alpha 1$ -syntrophin, a component of the dystrophin-associated protein complex (DAPC) (Au et al., 2008). However, this association has not been universally confirmed (Amiry-Moghaddam et al., 2003; Wakayama, 2010). This research is particularly relevant because reductions in AQP4 expression have been observed in other neuromuscular disorders and have been linked to the destabilization of the DAPC [see Figure 2] (Skauli et al., 2022).

Dystrophin, a key structural protein, bridges the cytoskeletal actin network to the extracellular matrix via the DAPC complex. In DMD-affected muscle, the absence of dystrophin leads to the destabilization of the DAPC, resulting in muscle membrane rupture and subsequent necrosis (Petrof et al., 1993).

Frigeri et al. (1998) were the first to observe a marked reduction of AQP4 in immunofluorescence staining of skeletal muscle from mdx mice. However, they initially questioned whether this reduction was directly related to DMD. Their subsequent investigations extended to the central nervous system (CNS) of mdx mice, where the results suggested that AQP4 was primarily involved in water reabsorption from extracellular fluid, blood, and cerebrospinal fluid, rather than being directly implicated in DMD pathology.

Interestingly, Frigeri and colleagues also noted differences in AQP4 levels between fast-twitch fibers in tibialis anterior (TA) muscle. However, this difference was not observed at the RNA level in mdx mice, leading to speculation that post-translational modifications might account for the observed discrepancies (Frigeri et al., 2001).

In contrast, the expression of AQP1 in skeletal muscle myofibers remains controversial. Several studies report negative immunostaining of AQP1 at the plasma membrane of skeletal myofibers, while others observed its presence in the endothelial cells of endomysial blood vessels (Frigeri et al., 2004; Wakayama, 2010).

Further supporting the link between AQP4 and muscle pathology, a study on rat tibialis anterior muscles found that AQP4 expression significantly decreased four days after denervation, coinciding with the onset of muscle atrophy (Au et al., 2008). Interestingly, in this same study, the downregulation of AQP4 was accompanied by a compensatory upregulation of AQP1.

Despite these findings, the roles of AQP4 and AQP1 in DMD remain ambiguous, with conflicting results from various studies. As of now, it is unclear whether

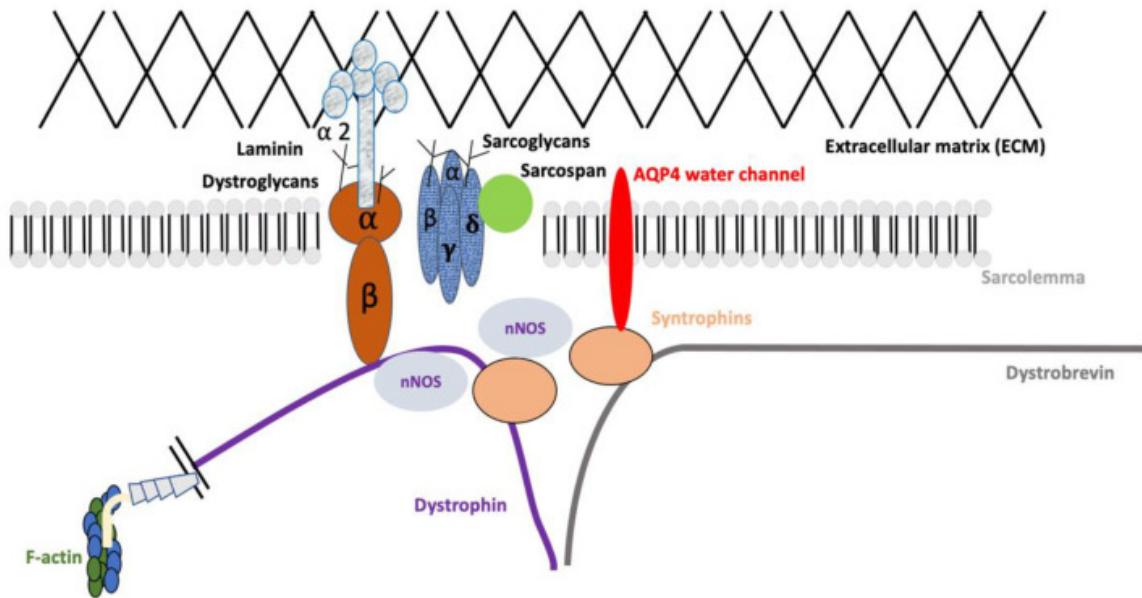


Figure 2: Representation of the DAPC complex in skeletal muscle. Adapted from “Assessing the Role of Aquaporin 4 in Skeletal Muscle Function,” by T. Aslesh, A. Al-aghabri, and T. Yokota, 2023, *International Journal of Molecular Sciences*, 24(2), p. 1489. Copyright 2023 by the authors. Licensed under CC BY 4.0.

either of these aquaporins can be definitively considered a biomarker for Duchenne muscular dystrophy (Aslesh et al., 2023).

2 Results & Discussion

In this section, results will be reported and compared with previous studies and/or clinically relevant features to assess the significance of the former.

Differential expression analysis was conducted using Wilcoxon’s test (for more details, refer to the Methods section). Our genes of interest are reported along with other disease-related or relevant genetic markers [see Table 2](Fortunato & Ferlini, 2023). Additionally, Benjamini-Hochberg FDR correction was applied to account for aberrantly small p-values, which are common in differential expression analysis(Korthauer et al., 2019). The significance thresholds used in this project are ($p < 0.05$ & $|\log_{2}FC| > 0.5$) in order to account for small changes in gene expression.

Interestingly, creatine kinase (CKM in muscle) was not found to be significantly differentially expressed ($p\text{-value} = 1.148 \times 10^{-1}$, $\text{FDR} = 1.292 \times 10^{-1}$). While this may seem surprising given that CK has traditionally been used as a biomarker for the diagnosis of dystrophinopathies, recent studies have elucidated that CK might not be suitable for disease monitoring (Aartsma-Rus et al., 2014; Fortunato & Ferlini, 2023). Considering that the patients in this study are all older than 6 years, we can conclude that CKM’s insignificant differential expression falls within expected parameters (Aartsma-Rus et al., 2014). Regarding MDH1 (Malate Dehydrogenase 1) and CAMK2A (Calcium/Calmodulin-Dependent Protein Kinase II Alpha), both involved in energy metabolism and signaling, processes that are

disrupted in muscle degeneration.

The aquaporin genes, AQP1 and AQP4, show contrasting results. AQP1 is significantly upregulated in the disease condition, while AQP4 shows a trend towards downregulation, although not statistically significant ($p\text{-value} = 0.06468$, $\text{FDR} = 0.07454$). These results partially resemble those obtained by Au et al. (2008)—in which AQP4 downregulation was compensated by AQP1 upregulation. The discrepancy in AQP4 expression—by not being significantly expressed—could be due to differences in disease stage, specific muscle types examined or due to sample size limitations.

On the other hand, pathway enrichment analysis elucidated which pathways were enriched under DMD conditions. For instance, those related to extracellular matrix (ECM) organization and cytoskeleton dynamics have been shown to be significantly altered in DMD [see Figure 4]. Moreover, inflammation-related pathways are also critical. The obtained results showcase enriched cell-substrate adhesion, ECM organization and small GTPase-mediated signal transduction pathways among others, reflecting the chronic inflammatory state observed during muscle degeneration(Ogundele et al., 2021; Pescatori et al., 2007). The lack of functional dystrophin also hinders the connection of the actin cytoskeleton to the extracellular matrix(Wilson et al., 2022).

Furthermore, amoeboid-type cell migration was also enriched. Such enrichment may relate to the altered behavior of muscle satellite cells, crucial for muscle regeneration in DMD(Boldrin et al., 2015). Once contrasted with clinical features, correlation matrices for AQP1 and AQP4 were created and pathway enrichment analysis of each of the sets of genes correlated to each aquaporin was conducted. While those genes highly correlated to AQP1 showcased enriched pathways related

Table 1: Results for differential expression analysis of genes of interest.

Gene	p_value	avg_log2FC	pct.1	pct.2	adjusted_p_value	FDR_BH
DMD	< 0.001	-0.7223411	0.553	0.824	< 0.001	< 0.001
CKM	0.1148	0.7954821	0.318	0.291	1	0.1292
AQP1	< 0.001	1.942773	0.066	0.015	< 0.001	< 0.001
AQP4	0.0647	-1.401034	0.018	0.024	1	0.0745
MDH1	< 0.001	0.1364925	0.061	0.041	0.7070	< 0.001
CAMK2A	< 0.001	0.5384431	0.118	0.059	< 0.001	< 0.001

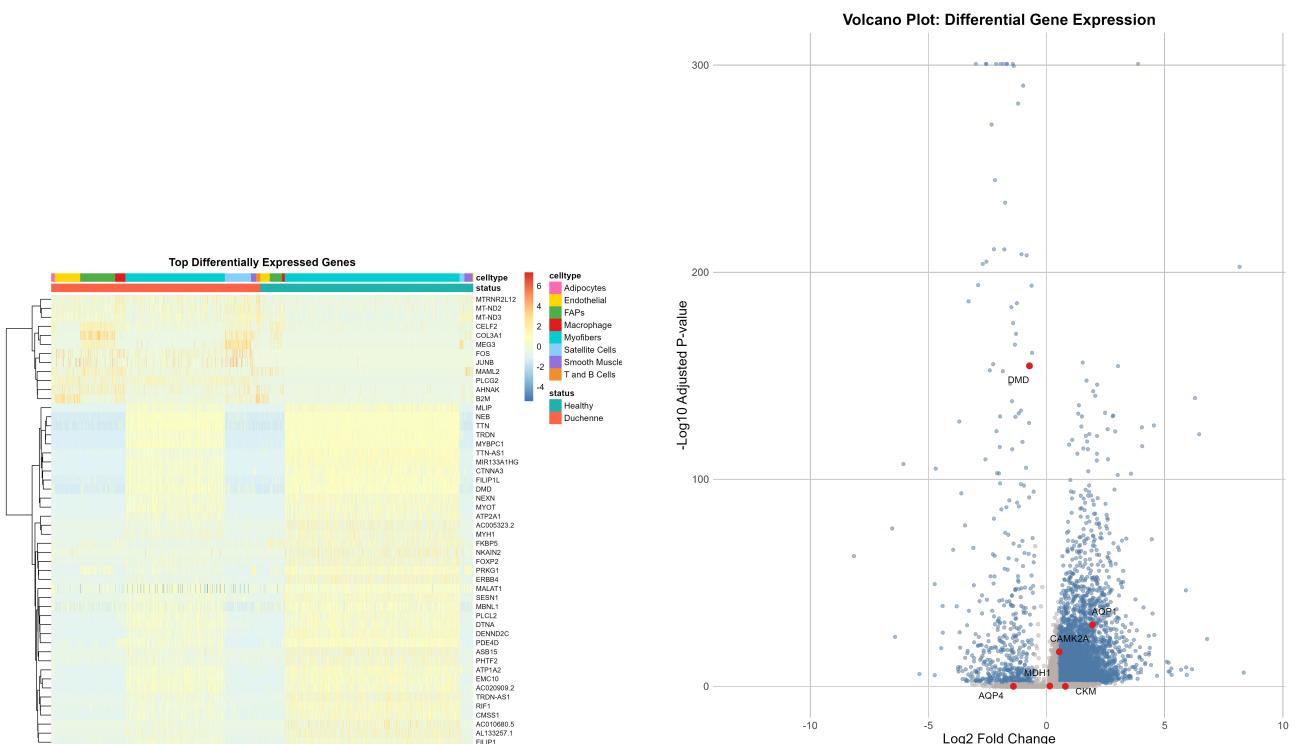


Figure 3: (a) Heatmap of the top 50 differentially expressed genes by condition status and cell type, (b) Volcano plot illustrating the top differentially expressed genes with genes of interest in red.

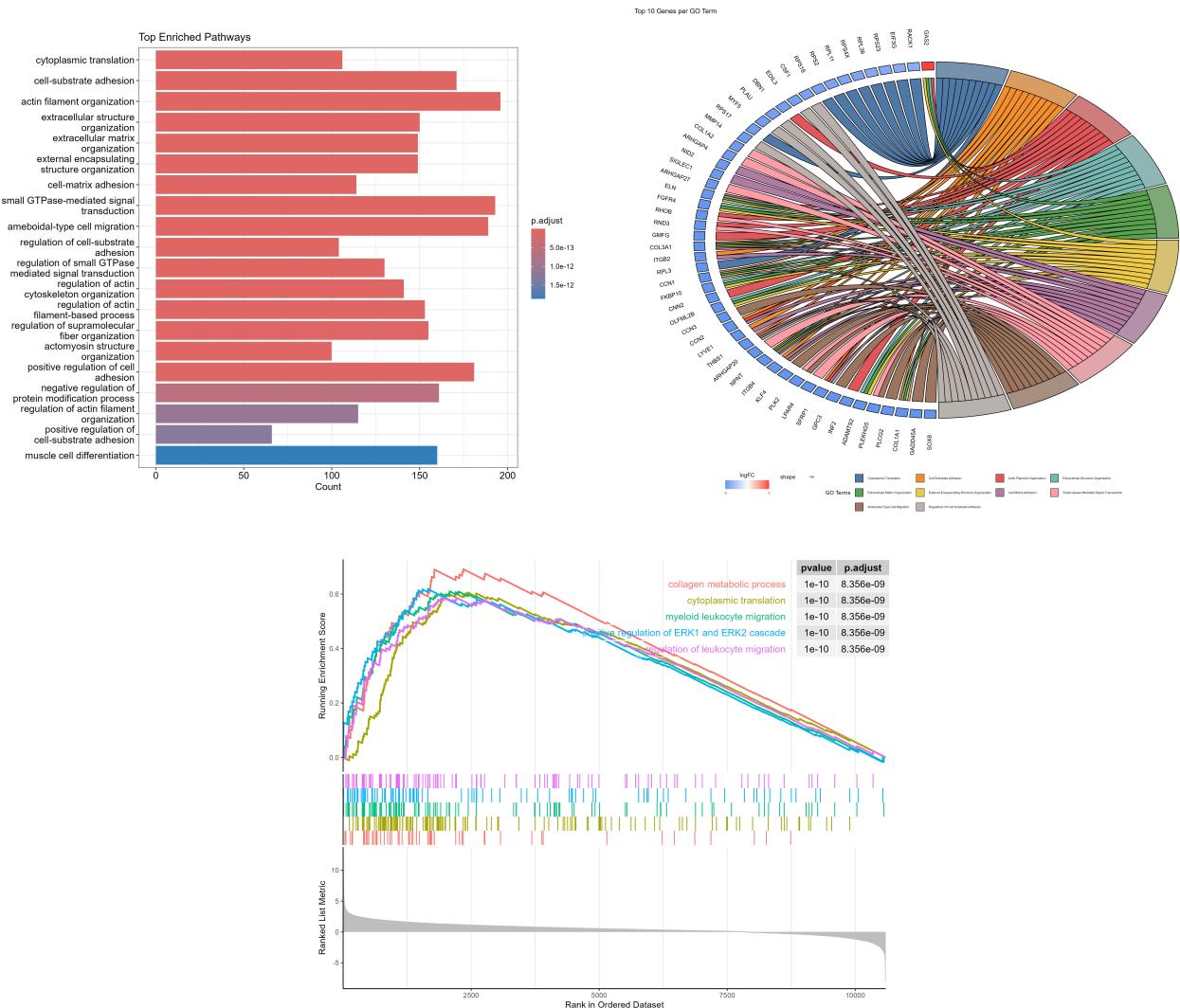


Figure 4: (a) Bar plot showcasing the top 10 enriched pathways, (b) Chord plot illustrating the top 10 genes by enriched pathway, (c) GSEA plot.

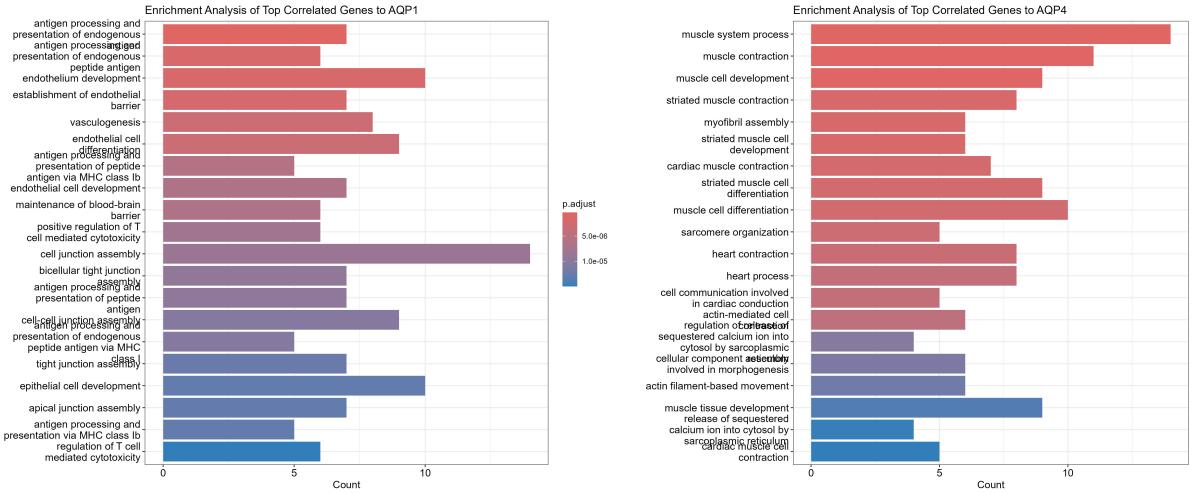


Figure 5: Enriched pathways from genes highly correlated to AQP1 and AQP4. Barplots are the result of enrichGO.

to MHC processes, antigen processing and vascular function; genes highly correlated to AQP4 were more likely to be related to muscle and heart function[see Figure ??].

AQP1 has been observed to be upregulated upon lipopolysaccharide stimulation in leucocytes and Mono-cytic THP-1 cells, which may explain the high correlation with genes participating in immune response-related pathways such as inflammation (da Silva & Soveral, 2021). In contrast AQP4's correlation with muscle cell differentiation, striated muscle development amongst others might be due to its association with the DAPC complex.

Moreover, AQP4 expression mainly focused in the myofiber subpopulations as opposed to AQP1 which was much more prominent in satellite cells — precursors of skeletal muscle cells. Given that satellite cells are involved in muscle repair and regeneration, the higher expression of AQP1 in these cells might suggest a role for this aquaporin in the regenerative process(Boyer et al., 2022; Jin et al., 2022). AQP4's higher expression in myofiber subpopulations might be related to its known role in water homeostasis in mature muscle fibers. This is consistent with previous literature mentioned earlier.

Although AQP1's expression was significantly upregulated, AQP4's was not significantly downregulated. However, it is plausible to conclude that the role of AQP1 and AQP4 are of great interest for Duchenne Muscular Dystrophy research. Hence, these findings warrant further investigation into the role of aquaporins in DMD pathophysiology. Studies integrating multi-omics data — not just snRNAseq— should be able to bring light to the intricacies of gene expression in DMD.

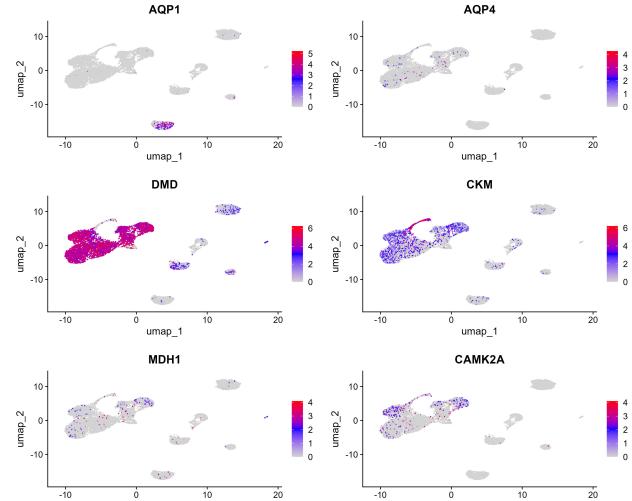


Figure 6: UMAP plot illustrating the gene expression of DMD, CKM, AQP1, AQP4, MDH1 and CAM2KA; previously discussed in Table 2

3 Methods

3.1 Data Collection and Preprocessing

In this project, I used the snRNA-seq data that the Nelson Laboratory at UCLA collected for two different projects (Nieves-Rodriguez et al., 2023; Scripture-Adams et al., 2022). A dataset of 3 healthy VL samples and 3 DMD VL samples was sent to me by colleagues at the Nelson Laboratory in the form of a Seurat object. However, I will cover a brief overview of the previous preprocessing they did on the raw RNA data. The procedure for the most recent publication was based in the procedure of the former publication.

Nieves-Rodriguez et.al. followed UCLA protocols and obtained muscle biopsies of fifteen healthy adults (ages ranging from 18 to 26) and eight ambulatory DMD patients with a confirmed nonsense mutation [for more

Table 2: Patient-sample characteristics.

Sample	Condition	Age	Sex
8100VL	Healthy	22	Male
8110VL	Healthy	22	Male
8108VL	Healthy	19	Male
1230VL	Duchenne	7	Male
8009VL	Duchenne	17	Male
8020VL	Duchenne	23	Male

information please refer to the original article]. Frozen skeletal muscle was then prepared for RNA extraction; healthy RNA with RIM > 7 and DMD RNA with RIM > 4 were selected for cDNA library preparation with ribosomal RNA depletion (Lee et al., 2020).

For bulk RNA sequencing, paired-end RNA sequencing reads were generated using the Illumina Novaseq 6000 S4. Reads were then aligned to GRCh38 using STAR (version 2.6.0) (Dobin et al., 2013).

Single nuclei were isolated from paired vastus lateralis (VL) and tibialis anterior (TA) frozen muscle samples from three healthy male subjects (Scripture-Adams et al., 2022). Libraries were prepared using the 10X Chromium Single Cell 3' v3 kit and sequenced on an Illumina NovaSeq 6000 S2 platform. The resulting single-nuclei RNA sequencing (snRNA-seq) reads were aligned to the GRCh38 reference genome using Cell Ranger, with initial clustering performed via k-means. Doublets were identified and removed using DoubletFinder, and nuclei with fewer than 200 unique molecular identifiers (UMIs) were excluded from analysis.

3.2 Analysis

Quality control metrics were initially assessed for the snRNA-seq data. The majority of samples contained between 500 and 1,500 unique genes, demonstrating consistency across identities and falling within acceptable ranges for single-nucleus RNA sequencing data(Cheng et al., 2023). As the dataset had already undergone preprocessing and quality control measures, including the consideration of mitochondrial gene percentages, no further analysis was deemed necessary in this regard. Moreover, log normalization and scaling had already been carried out prior to dataset retrieval. This "pre-cleaned" state of the data allowed for immediate progression to subsequent analytical steps.

Thus, the next step was to conduct exploratory data analysis. Cell type proportions were calculated and visualized by condition. As expected, diseased samples showcased significantly reduced levels of myofibers — due to cycles of degeneration and regeneration — and increased levels of adipocytes (Chemello et al., 2020; Fornander et al., 2021). Pearson's Chi-square test was used to assess whether the changes in cell population proportion were random or significant.

Proceeded with EDA by conducting PCA and UMAP analysis. These would allow for clustering — which is useful for finding differentially expressed genes in each

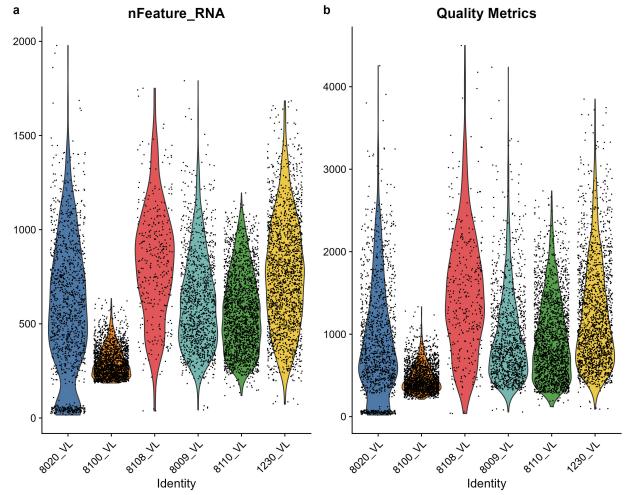


Figure 7: Quality control metrics for snRNA-seq data. **(a)** Distribution of unique genes detected across different sample identities. **(b)** Count of RNA molecules detected per sample.

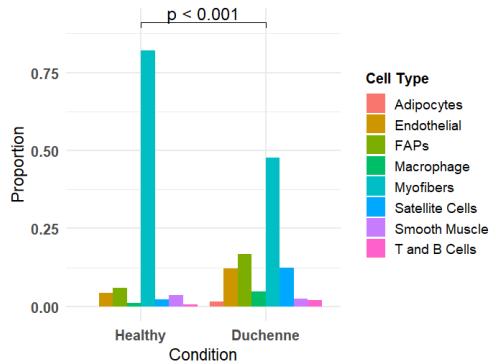


Figure 8: Cell type proportions. The populations present are: myofibers, macrophages, endothelial, satellite cells, fibroadipogenic progenitor cells (FAPs), smooth muscle, T and B cells and adipocytes. Proportions significantly differ between healthy and DMD groups (p -value < 0.001).

of the subpopulations in the samples — and to easily visualize the distribution of healthy and diseased cells in the dataset.

Principal Component Analysis showcases two clusters, differentiable by differing status. With regards to the UMAP visualizations, the resulting clustering confirms a successful cell type identification. The visualization also suggests that while cell types are largely consistent between conditions, there are disease-specific changes in gene expression, especially in myofibers(Frigeri et al., 1998). Deeper analysis regarding PCA illustrated which were the top contributing genes to PC1 and PC2 [see Table 3].

Reached this point, aquaporin analysis was conducted. By first assessing which aquaporin channels were present in the samples I was able to identify cell populations that were more prone to expressing these channels. The aquaporins present were: AQP1, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, AQP8, AQP9, AQP10

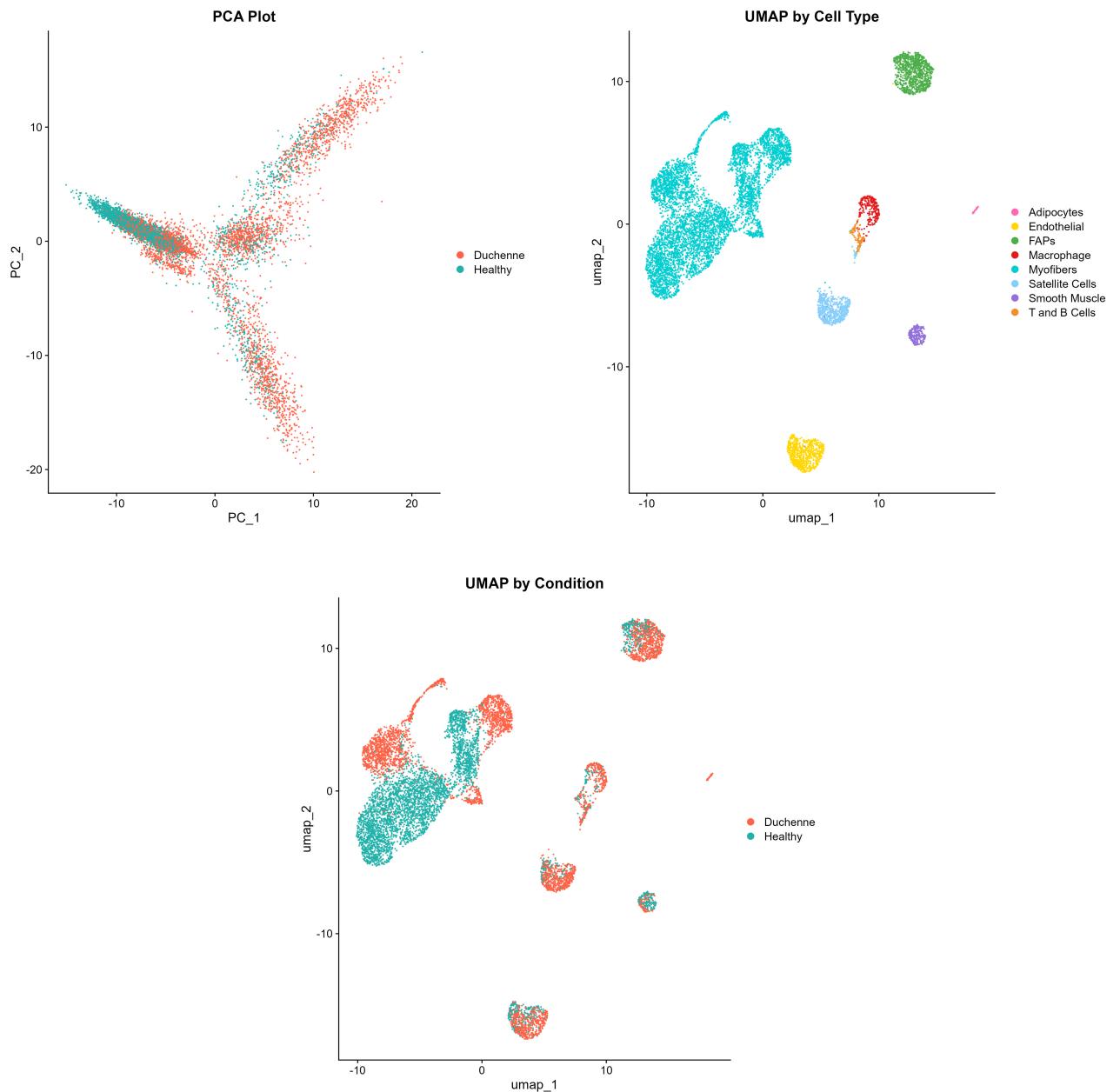


Figure 9: A mosaic layout displaying three plots: (a) PCA plot, (b) UMAP visualization by cell types, and (c) UMAP visualization by status.

Table 3: Top features and their weights for PC1 and PC2

PC1		PC2	
Feature	Weight	Feature	Weight
ATP2A1	-0.1391	CD74	-0.1242
MYH1	-0.1261	AQP1	-0.1185
MIR133A1HG	-0.1243	EGFL7	-0.1085
TNNT3	-0.1201	VWF	-0.1048
FILIP1L	-0.1138	PECAM1	-0.0988
DENND2C	-0.1124	PTPRB	-0.0983

and AQP11 [see Figure 10].

Statistical analysis of differential expression (DE) was conducted using the Seurat package (v.5.1.0). For differential expression analysis, the Wilcoxon Rank Sum test was used due to its simplicity and ability to control FDR— Benjamini-Hochberg— on large sample-size data(Li et al., 2022; Pullin & McCarthy, 2024). On the other hand, enrichR and enrichplot (v.3.2 and 1.24.4, respectively), were used for pathway enrichment analysis along with org.Hs.eg.db(v.3.19.1). For all enrichment analysis conducted in the project default settings for the enrichGO function were used: *pAdjustMethod = "BH"*, *pvalueCutoff = 0.05*, *qvalueCutoff = 0.05*.

The philosophy underlying the methodology used for this project is as follows: by first determining which genes are differentially expressed, it is possible to determine whether AQP1 and AQP4 were differentially expressed. Regardless of the results, pathway enrichment analysis is conducted on the top-differentially expressed genes($p < 0.05$ & $|\log_{2}FC| > 0.5$) to assess which pathways are enriched due to Duchenne muscular dystrophy and check whether there is any relationship with clinical features of the disease. This aims to identify disease-relevant biological processes beyond individual gene changes(Miyoshi et al., 2023). GSEA was also conducted to avoid differential cut-off selection issue(Kotelnikova et al., 2012; Subramanian et al., 2005). This post-hoc analysis to DE analysis serves as a "checkpoint" for guaranteeing the validity of the data by contrasting with clinically relevant features of DMD and thus infer if there might have been errors while handling the data.

Following this analysis, correlation matrices were created for the top 100 genes correlated to AQP1 and AQP4. This approach is supported by previous studies on aquaporins, which have used correlation matrices to analyze water transport and protein interactions(Hashido et al., 2007). For instance, Hashido et al. used osmotic permeability matrices to examine correlated motions of water molecules in different aquaporin channels, including AQP1 and AQP4. While their focus was on water molecule correlations rather than gene correlations, the principle of using matrices to understand relationships in aquaporin function is analogous to our approach.

Thereafter, further aquaporin-specific pathway enrichment analysis is conducted to assess any new clinical

features and compare with published bibliography and clinical records.

4 Acknowledgements

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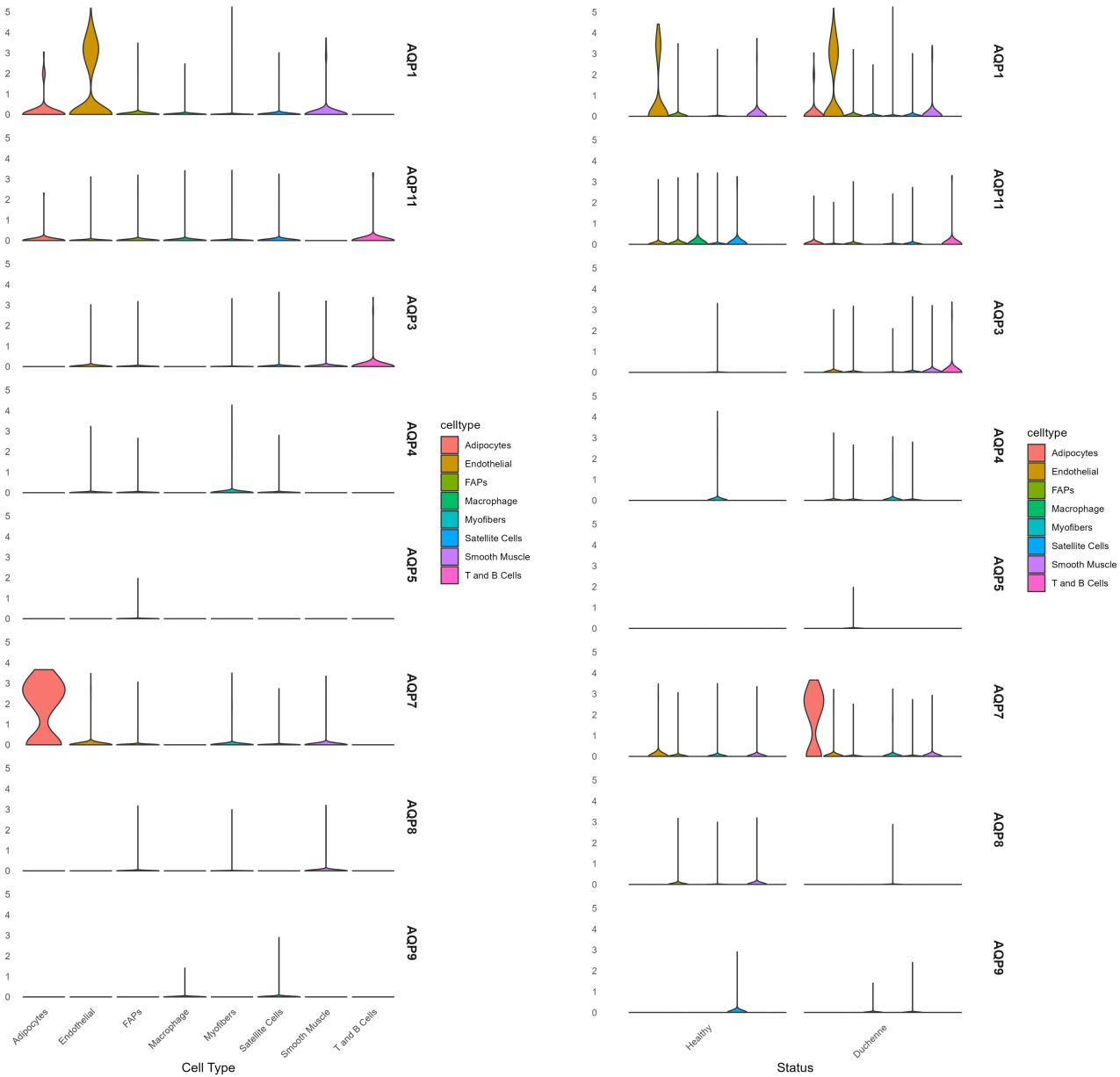


Figure 10: The plots showcase the expression of aquaporin genes. (a) Violin plot of aquaporin expression by cell type, (b) Violin plot of aquaporin expression by status.

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