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Original Article

Elevated plasma complement components in facioscapulohumeral dystrophy

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Abstract

Advances in understanding the pathophysiology of facioscapulohumeral dystrophy (FSHD) have led to several therapeutic approaches entering clinical trials and an increased need to develop biomarkers of disease activity and progression. Multiple prior studies have shown early elevation of RNAs encoding components of the complement pathways and relatively widespread activated complement complexes by immunodetection in FSHD muscle. The current study tested plasma from two independent cohorts of FSHD and control subjects and found elevated complement components in both FSHD cohorts. Combining subjects from both cohorts identified complement factors that best distinguished FSHD and controls. Within the FSHD group, a subset of subjects showed elevation in multiple complement components. Together these findings suggest the need for future studies to determine whether measurements of complement activation can be used as a non-invasive measurement of FSHD disease activity, progression and/or response to therapies. In addition, with the ongoing expansion of complement therapeutic approaches, consideration for precision-based targeting of this pathway is appropriate.

Introduction

Facioscapulohumeral dystrophy (FSHD), the third most common muscular dystrophy, is caused by the mis-expression of the DUX4 transcription factor in skeletal muscle (1). DUX4 is normally expressed at the four-cell cleavage stage in human embryos where it activates part of the first wave of zygotic gene expression (2,3). While the facial and upper-extremity muscles are frequently the first affected, FSHD ultimately affects nearly all skeletal muscle groups. The histopathology shows interstitial fibrosis, signs of regeneration with variable fiber size and central nuclei, and a patchy perivascular distribution of focal inflammation (4–6). A model is emerging that suggests inflammation is both an early characteristic of FSHD and a marker of disease progression. Magnetic resonance imaging (MRI) studies have identified an abnormal T2-STIR signal as an early sign of muscle involvement, suggesting that inflammation and/or edema is an early pathological finding. Biopsies of short tau inversion recovery (STIR)-positive FSHD muscle showed infiltration with CD4 and CD8 T-cells and presence of elevated cytokines (7). Subsequent studies confirmed the association of MRI STIR signal with a higher probability of inflammation in the muscle biopsy and with the expression of RNAs induced by DUX4 (8,9). These and other studies (10–13) suggest a model of disease progression from relatively normal muscle histology and MRI progressing to a T2-STIR signal and inflammation, and ultimately to fatty infiltration and conversion to a T1 MRI signal. Coincident with the MRI progression and increased inflammation in the histopathology, muscle biopsy RNA sequencing also shows increased RNAs associated with DUX4 expression, inflammation, extracellular matrix and other markers of disease activity (9). It is notable that FSHD muscles with a normal MRI signal and relatively mild features on histopathology express low levels of these same RNA markers of disease activity, indicating a that there is a prolonged phase of low-level disease activity and raising the possibility that biomarkers might be identified that correspond to this widespread low-level disease activity (8,9).

Several studies suggest that activation of the complement system is an early feature of FSHD muscle, and complement component RNAs become progressively elevated with disease progression. Sarcolemmal

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complement deposits were shown present in non-necrotic muscle FSHD muscle fibers using an antibody that recognizes a component of the activated membrane attack complex (MAC) (14). Subsequent studies reported elevated complement component RNAs in T2-STIR positive muscles (15) and in both the more severely affected biceps muscles and the more mildly affected deltoid muscles (16). Additionally, perivascular and sarcolemmal deposits of the activated MAC complex were also found in FSHD muscle with normal MRI and near normal histopathology (8), and a microdialysis study identified elevated complement factor D in both STIR+ and STIR– muscles (17). Together these studies suggest a potential early role of complement activation in the development of cellular injury in FSHD.

The early and widespread activation of the complement system in FSHD muscle makes plasma complement levels a possible measure of FSHD disease and/or activity. Indeed, in a screen for serum biomarkers that used an immunoassay, C3 levels were elevated in FSHD (18), and in an aptamer based screen C3b was elevated in one of two FSHD cohorts (19). In the current study, we used a panel of enzyme-Linked Immunosorbent assay (ELISA) based assays to multiple components of the major complement activation pathways to determine whether one or more component showed elevation in FSHD compared to controls. A subset of complement components of the classical, alternative and terminal pathways showed strong trends towards elevation in the FSHD samples, and C3 showed significant elevation in both cohorts. Further studies will be necessary to develop robust assays for complement activation in FSHD and ultimately to determine whether measurements of plasma or tissue complement activation correlate with aspects of disease progression and/or response to therapeutic intervention.

Results

Complement assays in first cohort of FSHD and control plasma samples identifies complement components elevated in FSHD plasma

We analyzed complement levels in 24 FSHD subjects and 12 controls (Supplementary Material, Table S1). The age of FSHD subjects ranged from 18 to 72 with a mean 52 (+/−14 sd) and a sex distribution of 50% male and 50% female; similarly, the control ages ranged from 12 to 63 with a mean 45.3 (+/−15.1 sd) and a sex distribution of 50% male and 50% female. Complement levels in fresh frozen plasma samples were determined by Exsera BioLabs. The tested complement components span most of the complement cascade (Fig. 1), and included six components of the classical/lectin activation pathway (C1q, C4, MBL, C4a, C2, C4b), four of the alternative pathway (Factor H, Factor I, Factor D, Factor B) and four of the terminal pathways (C3, C5, C5a, sC5b-9).

Comparison of each complement component in the FSHD group to the control group showed that the levels of C2, C3 and sC5b-9 were significantly elevated in the FSHD group compared to the control group (two way *t*-tests *P*-value < 0.05) (Fig. 2a and b). Factor D and C4a showed a moderate elevation in FSHD (*P*-value < 0.2) with a few FSHD samples exhibiting levels higher than the ranges of controls (see Fig. 2b). Although this analysis showed elevation of specific complement components in the FSHD group, using the entire panel of complement assays hierarchical cluster analysis (see Methods) did not distinguish the FSHD and control samples (Supplementary Material, Fig. S1).

Complement assays in a second cohort confirms elevation of complement components

We next analyzed complement component levels in plasma from a second cohort of 41 FSHD subjects and 17 controls (Supplementary Material, Table S2) measuring the same complement components with the addition of Bb. The age of FSHD subjects ranged from 19 to 72 with a mean 55.2 (+/−13 sd) and a sex distribution of 39% male and 61% female; similarly, the control ages ranged from 18 to 71 with a mean 45.8 (+/−15.9 sd) and a sex distribution of 35% male and 65% female (Supplementary Material, Table S2).

Comparison of each complement component in the 41 subjects in the FSHD group to the 17 in the control group showed significant elevation of C3 and C4b in the FSHD group (Fig. 2c). C4a, C5a, SC5b-9 and Factor D showed a trend toward elevation in the FSHD samples in both the first and second cohort (Fig. 2c and d), suggesting activation of C3 and the terminal pathway components through both the classical pathway involving C4a and C4b and the alternative pathway involving Factor D. In contrast to the first cohort, C2 showed a significant decrease in FSHD subjects in the second cohort, although the reason for this is not known, the C2 levels in the second cohort controls were higher than in the first cohort controls and showed greater variability (Supplementary Material, Fig. S2a).

Twenty of the 41 cohort 2 FSHD subjects provided a second plasma sample at a 3 month follow-up visit that was analyzed at the same time as the entire cohort 2 sample set. Although there was some variation, most of the complement levels were generally stable during this 3 month interval (Supplementary Material, Fig S2b).

Analysis in a combined cohort identifies complement factors that merit future studies as candidate biomarkers in FSHD

To explore the complement components that best distinguish FSHD from controls, we analyzed an expanded data set that combined the 41 FSHD second cohort first plasma samples with 20 new plasma samples collected from cohort 1 at a follow-up visit, and compared them to the 17 control samples from the second cohort (all run in the same second cohort assay group; Supplementary Material, Table S3, also see Supplementary Material, Table S4 for a comprehensive table with all subject data). We identified a subset of complement components

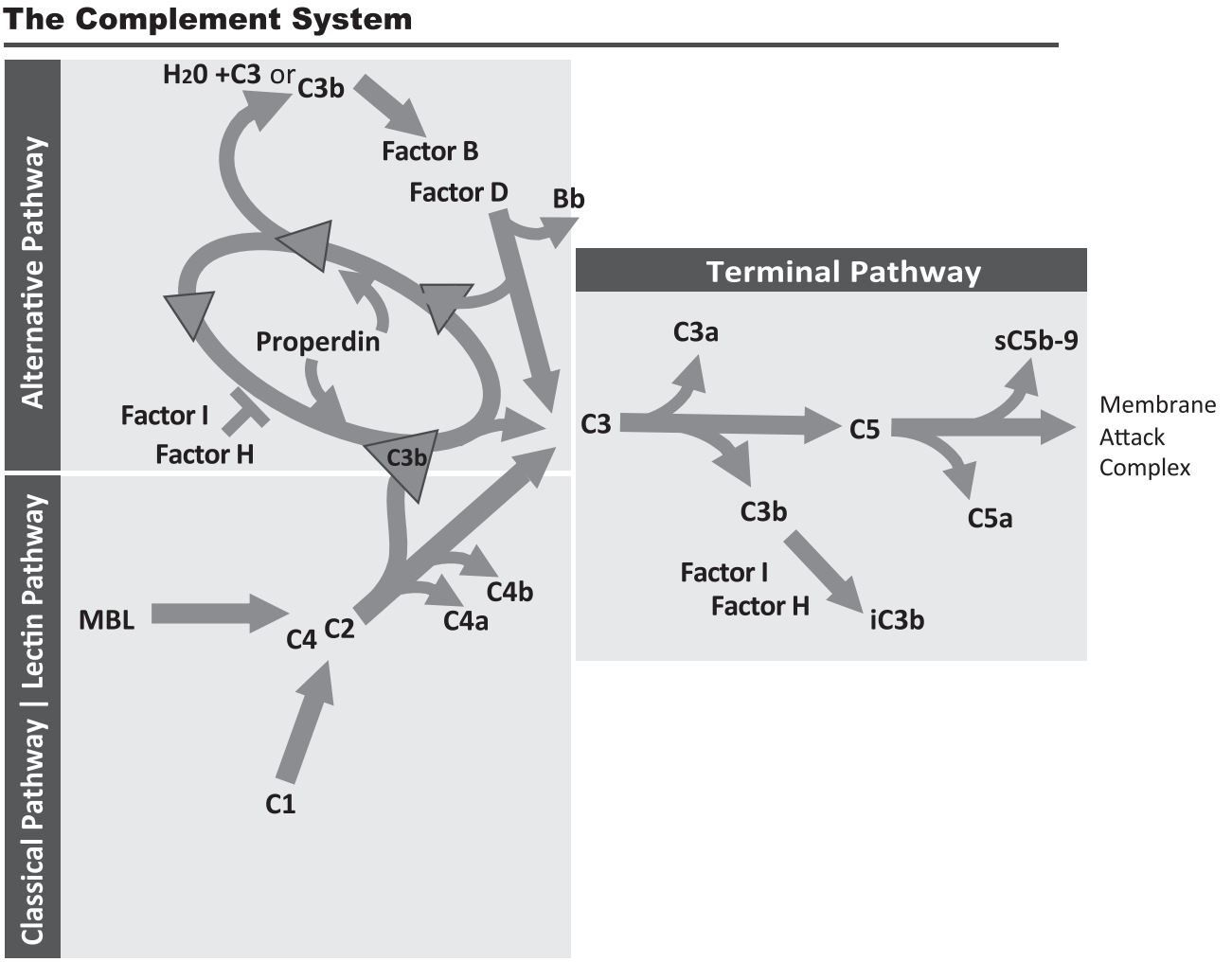

Figure 1. Complement cascade system. Diagram showing the complement components of the classical, alternative and terminal pathways of the complement system.

Table 1. Members of the ReSolve Network listed as collaborators for this study

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University of Washington	Leo Wang (PI), Matt Preston (Co-I), Cat Kieu (Evaluator), Laura Johnstone (Evaluator), Corrie Moreau (Coordinator)
University of Utah	Russell Butterfield (PI), Melissa McIntyre (Evaluator), Amelia Wilson (Evaluator), Sarah Moldt (Coordinator)
Kennedy Krieger Institute	Doris Leung (PI), Kathryn Wagner (Co-I), Jessica Nance (Co-I), Nikia Stinson (Evaluator), Mary Yep (Coordinator)
University of California, Los Angeles	Perry Shieh (PI), Francy Shu (Co-I), Christy Skura (Evaluator), Jennifer Huynh (Coordinator)
Virginia Commonwealth University	Nicholas Johnson (PI), Amanda Butler (Evaluator), Jodie Howell (Coordinator)
Radboud University	Karlien Mul (PI), Baziel van Engelen (Co-I)
NEMO Clinical Research Center	Valeria Sansone (PI), Elena Carraro (Co-I), Maria Chiara Frisoni (Evaluator), Alessandra DiBari (Coordinator)
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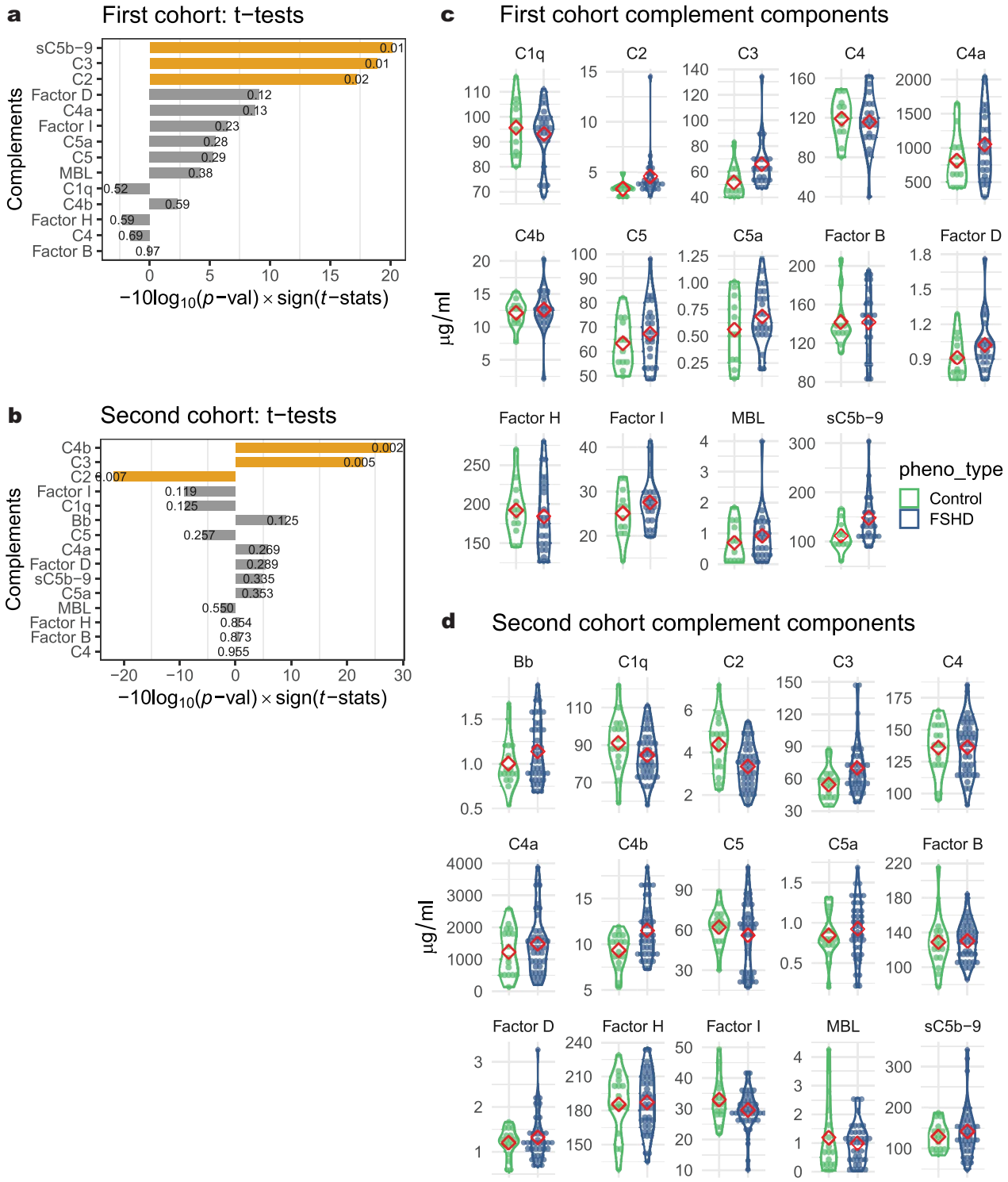

Figure 2. Complement profile in first and second cohorts. (**a** and **b**) First cohort complement profiles. (a) Complement level *t*-tests comparing FSHD and control samples. The horizontal bar depicts the scaled *P*-value and sign of *t*-statistics. Orange indicates *P*-value < 0.05. (b) Violin plots showing the distribution of the complement levels in FSHD and controls. Green and blue dots represent the control and FSHD samples, respectively; red dots are the mean values in each group. (**c** and **d**) Complement profiles in the second cohort. (c) Complement level *t*-tests comparing FSHD and control samples. The horizontal bar depicts the scaled *P*-value and sign of *t*-statistics. Orange indicates *P*-value < 0.05. (d) Violin plots showing the distribution of the complement levels in FSHD and controls. Green and blue dots represent the control and FSHD samples, respectively; red dots are the mean values in each group.

with correlated variation between the FSHD and control groups (Supplementary Material, Fig. S3a-f) and used the four showing highest association with FSHD (C3, C4b, Factor D and sC5b-9). Based on this panel, clear trends for elevated expression in FSHD were evident (Fig. 3a) and hierarchical clustering showed a subset of FSHD subjects with higher expression than most controls in multiple complement components (Fig. 3b), suggesting that broad dysregulation and activation of complement pathways might be used to identify subsets of FSHD individuals with higher complement plasma levels for future correlation with measures of disease activity or progression.

We assigned a single composite complement score for each individual subject based on the sum of the z-scores for each component of the panel (Supplementary Material, Table S5) and used a waterfall and box-whisker plots to visualize relative levels of the z-score for the complement factors in controls and FSHD individuals, demonstrating the complement score based on these four complement factors significantly distinguished the FSHD group from the controls (Fig. 3d).

Together, these findings suggest that measuring select components of plasma complement in plasma might have utility for identifying subsets of FSHD subjects that can be segregated for subset analysis on progression or response to therapies in future studies.

Neither the clinical severity scores nor the number of D4Z4 repeats correlate with complement levels

This study was not designed to determine whether the Clinical Severity Score or D4Z4 repeat number correlated with complement levels; however, for completeness we performed Pearson's correlation tests on the 53 FSHD samples from the combined cohort. The correlation between CSS and complement levels was low: 0.19 between CSS and the composite z-scores based on the four complement components, and less than 0.21 between CSS and each of the 14 complement components. The D4Z4 repeat number was not associated with the composite complement score (Pearson = −0.13). Neither MRI nor muscle biopsies were performed in this initial study, so these comparisons cannot be made.

Discussion

The recognition that FSHD is caused by the mis-expression of the embryonic transcription factor DUX4 in skeletal muscle has led to several therapeutic approaches entering clinical trials (20,21). The advancement of therapies to clinical trials has increased the need for measures of disease activity and progression that can be further tested as candidate biomarkers for clinical trials. In addition, identification of pathways that mediate tissue damage in patients with FSHD can provide additional therapeutic options. The current study shows a reproducible and significant elevation of complement C3 in FSHD cohorts compared to controls, and reproducible elevated trends in several other complement components and activation fragments in these cohorts. It is notable that while the majority of FSHD subjects show complement levels in the same range as the control subjects, thereby precluding the use of complement as a general biomarker for FSHD, a subset of FSHD subjects show elevation of multiple complement components. This study was not designed to test whether elevations in plasma complement in this subset correlate with disease severity or progression; however, the current results indicate the need for future studies to test these correlations.

The current study used a broad and representative panel of complement components to show elevations of some components from the three main complement pathways (classical, alternative, terminal) in FSHD. Future studies might focus on larger cohorts and a subset of activated complement components, such as the subset of four factors determined most discriminative in the analysis of the full data-set in this study. A recent study showed elevated levels of interleukin 6 (IL6) in FSHD that correlated with disease severity (22), raising the possibility of including both complement and IL6 measures in future studies. Furthermore, although a multitude of complement assays exist for clinical development, there is both a need and ongoing efforts to improve their sensitivity and robustness (23). One example of a possible improvement for future studies is the use of a recombinant CR2 (complement C3d receptor 2) to enhance detection of iC3b and C3dg in plasma (24).

Another approach to consider is non-invasive monitoring of organ-specific complement activity in FSHD. For example, in other preclinical models of disease, radio-isotope conjugated CR2 was used with MRI to detect disease activity in a lupus model of nephritis (25) and with single-photon emission computed tomography (SPECT/CT) to detect complement activation in a mouse model of myocardial ischemia-reperfusion injury (26). Also, a radiolabeled monoclonal antibody that recognizes the tissue bound iC3b and C3d, but not the native circulating C3 or C3b, was used to detect pulmonary granulomas by SPECT/CT imaging in a mouse model of tuberculosis (27). Such approaches might be useful to monitor disease progression and response to therapy specifically in individual skeletal muscles in FSHD.

The complement system is regulated by the balance between activation and regulatory mechanisms (28) and there are several possible mechanisms for complement activation in FSHD muscle. It is notable that evidence in FSHD exists for engagement of each of the three activation pathways as well as the amplification loop. Although serum antibodies against antigens expressed in FSHD muscle have not been identified (29), the classical pathway could be engaged by natural antibody recognition of neoepitopes expressed on injured myocytes or the release of intracellular components that engage C1q (30). The lectin pathway can similarly be activated by

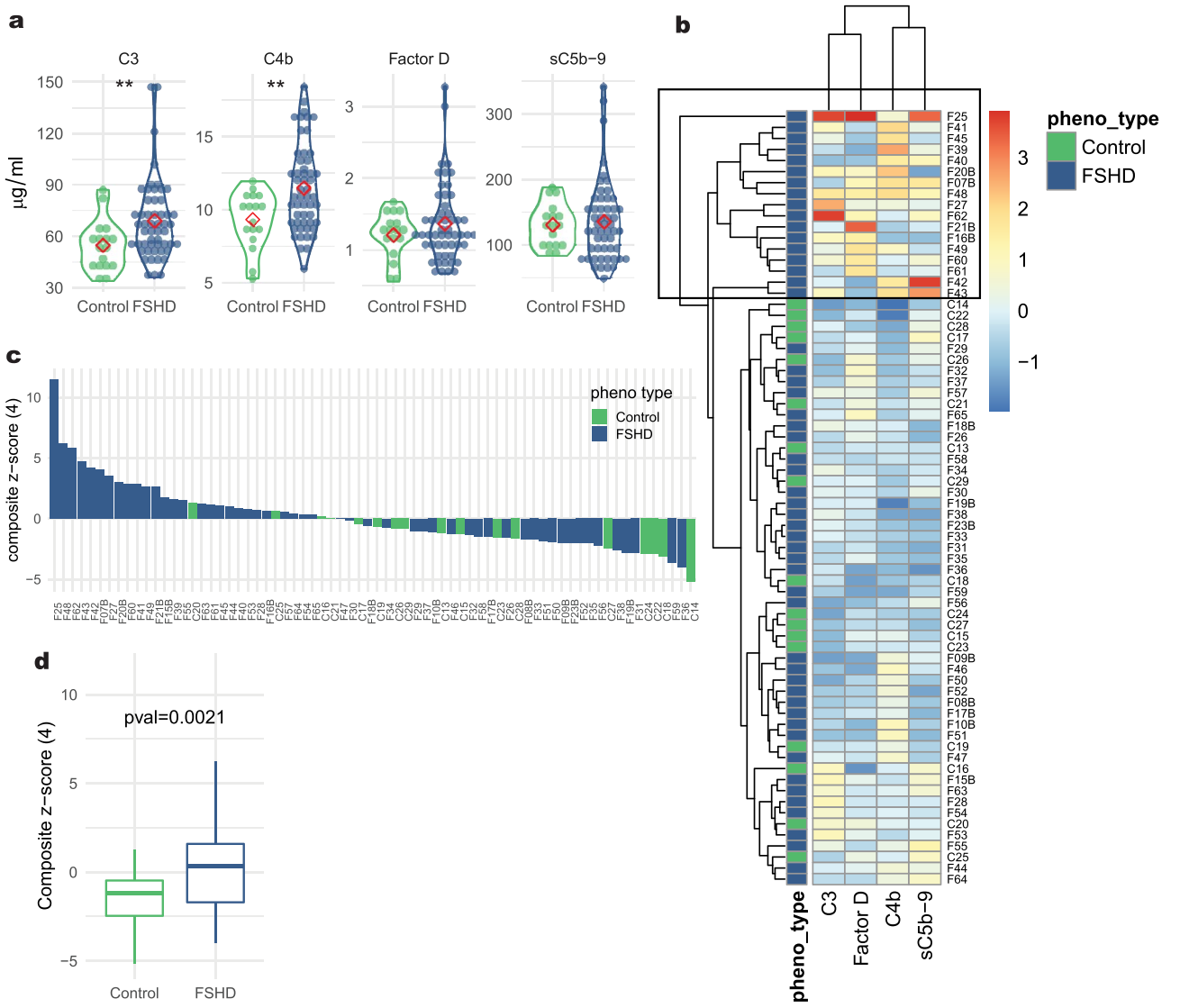

Figure 3. Complement levels and per-sample composite scores of the combined plasma samples from two cohorts. The combined samples include 20 s plasma samples from the first cohort and 41 first plasma samples from the second cohort. (**a**) A violin plot showing the distribution of the selected complement components in FSHD and controls. Green and blue dots represent the control and FSHD samples, respectively; red dots are the mean values in each group. **P-value < 0.005; P-values of Factor D and sC5b-9 are 0.15 and 0.67, respectively. (**b**) Hierarchical cluster-based heatmap of the complement levels. The top 17 samples enclosed in the rectangle are FSHD subjects displaying elevation in one or more pathways. (**c**-**d**) Waterfall plot (c) and boxplot (d) of per-sample composite z-score, based on the panel of four components (C3, C4b, Factor D and SC5b-9), showing the relative levels of the combined z-score for each subject.

pattern recognition engagement of factors generated during injury (31). Evidence also exists that a decrease in membrane regulators, either endogenously expressed or based on relative Factor H binding, can allow the alternative pathway to initiate C3b deposition (32). In addition, skeletal muscle endogenous complement activation might also have an early role. RNA sequencing datasets from human skeletal muscle cells with induced expression of DUX4 show induction of RNAs for several complement components, e.g. C1S, C5, C3 and others. Although most are modestly induced by DUX4, this nonetheless demonstrates a possible cell autonomous contribution to complement activation. Once initiated, the complement system might promote immune cell infiltration that would further spread complement activation. This, and the potential for activated complement on the sarcolemma of one myofiber to damage the adjacent myofiber might provide important mechanisms for how DUX4 expression and muscle damage slowly amplify and spread through the affected muscle.

Finally, the demonstration of activated complement in this and prior studies suggests that complement inhibiting therapies should be considered, particularly if plasma or imaging techniques can be advanced for more robust and tissue-specific monitoring of complement activity in FSHD muscle. Currently, monoclonal antibodies that inhibit the activation of C5 (eculizumab and ravulizumab) are used for the treatment of myasthenia gravis, paradoxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome and neuromyeitis

optica; and C1INH (C1-inhibitor) (either recombinant or plasma purified) is used for the treatment of hereditary angioedema. In addition, derivatives of the C3 activation inhibitor compstatin are reaching the clinic for use in complement-mediated diseases (33). Although premature to suggest complement-directed therapies in FSHD, their successful deployment in other diseases associated with complement activation suggests that the findings in the current study should be extended and might ultimately contribute to future therapies.

Materials and Methods

Plasma sample acquisition

All plasma samples from individuals with FSHD were obtained from four sites participating in an ongoing NIH funded U01 grant titled 'Clinical Trial Readiness to Solve Barriers to Drug Development in FSHD (ReSolve)' which is a 24 months observational study in FSHD (34). The study protocol was approved by the Kansas University Medical Center's central IRB. Normal control samples were collected from volunteers at the University of Rochester Medical center under a separate local IRB-approved protocol. Study subjects with FSHD were 18–75 years old with genetically confirmed FSHD1, with symptomatic limb weakness and able to walk 30 feet without support of another person. Individuals with cardiac or respiratory dysfunction, regular use of anabolic or catabolic agents or use of experimental drug in and FSHD trial were excluded. Normal control individuals had no muscle weakness and met the same exclusion criteria as study subjects with FSHD. All study subjects signed a corresponding consent form. For plasma extraction, whole blood was collected in 10 mL EDTA tubes and stored on wet ice and centrifuged within 1 h. The samples were centrifuged either at 4°C or at room temperature for 10 min at ~2700 PRM (1300 ± 100 x g). Immediately after centrifugation the upper layer (plasma) was transferred into labeled cryovials in 0.5 mL aliquots and stored at −80°C. A total of 100 FSHD plasma samples and 32 control samples were collected for the ReSolve study.

Complement analysis

Complement levels were assessed by either multiplex Luminex or single ELISA assay methods. For the multiplex analysis, the human complement bead-based xMAP technology (Luminex Corp, Northbrook IL) and commercially available kits (EMD Millipore, Milliplex Map, Burlington, MA) were used to measure 13 complement proteins, spanning all three activation arms and the terminal pathway of complement. Measurements were made on a MagPix Luminex instrument. The Millipore Panel #1 was used to measure C2, C4b, C5a, C9, FD, MBL and Factor I (FI). Panel #2 was used to measure C1q, C3, C4, Factor B (FB) and Factor H (FH) that was measured as a single plex. In addition, the complement activation markers Bb, C4a, C3a and the, sC5b-9 were measured by ELISA (Bb, C3a, sC5b-9: Quidel Corp, San Diego CA and C4a by OptiEIA BD Biosciences, Franklin Lakes, New Jersey). All testing methods had been optimized and validated within Exsera BioLabs, a College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA) certified laboratory.

All analysis was performed in duplicate with the resulting mean values report. For the multiplex Luminex data, the mean fluorescent intensity was the raw value and for the ELISA analysis the raw values were optical density. Standard curves and a four-parameter parametric curve fit were utilized to calculate the absolute quality in ng/mL or μg/mL, as appropriate. Three quality controls (QC) were included in each run, including at least one laboratory developed and characterized QC.

Data analysis

We used *t*-tests to perform hypothesis testing on whether the complement level is elevated in FSHD. For the use of multivariate analysis (clustering and principal components analysis (PCA)) and visualization, we standardized the scale of complement levels and used the z-score as our normalized matrix, i.e. for each complement i and sample j, the z-score is given by $Z_{i,j} = (x_{i,j} - \overline{X_j})/\mathbf{SD}_j$, where $x_{i,j}$ is the complement level. We applied hierarchical cluster analysis on the panel of seven complement components to find clusters of FSHDs showing elevation and used PCA to visualize the variation of sample distance among FSHD and controls. All the analyses and figures were made by using R-4.0.3 and the tidyverse packages. The R codes (both Rmd and R files) and data sets are available on our github repository (see Data and code availability section).

Data and Code Availability

Our github repository (https://github.com/FredHutch/Wellstone_Plasma_Complement_in_FSHD) is public and contains the complete datasets (in both excel and R-compatable format) and R codes. To support the computational reproducibility, we also built a github page (https://FredHutch.github.io/Wellstone_Plasma_Complement_in_FSHD) by the *bookdown* package to include the detailed analysis workflow and automatically executable R codes that produce all the statistics and figures in this manuscript.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. L.H.W., S.V.D.M., R.T., J.S., S.J.T. consult for pharmaceutical companies interested in clinical trials design for FSHD. V.M.H. has income from complement therapeutics royalties, consulting and equity.

A.F.A. consults for pharmaceutical companies but none with clinical trials for FSHD and is the Director of Exsera BioLabs. No company funded any part of this study or had any influence in its design, execution or publication. The other authors declare no conflict of interest.

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