Comprehensive transcriptomic analysis shows disturbed calcium homeostasis and deregulation of T lymphocyte apoptosis in inclusion body myositis

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Keywords: inclusion body myositis, calcium, differential expression, differential splicing, T cells

31 **Abstract**

32 Objective: Inclusion body myositis (IBM) has an unclear molecular etiology due to the co-existence
33 of characteristic cytotoxic T-cell activity and degeneration of muscle fibers. Using in-depth gene
34 expression and splicing studies, we aimed at understanding the different components of the molecular
35 pathomechanisms in IBM.

36 Methods: We performed RNA-seq on RNA extracted from skeletal muscle biopsies of clinically and
37 histopathologically defined IBM (n=24), tibial muscular dystrophy (n=6), and histopathologically
38 normal group (n=9). In a comprehensive transcriptomics analysis, we analyzed the differential gene
39 expression, differential splicing and exon usage, downstream pathway analysis, and the interplay
40 between coding and non-coding RNAs (micro RNAs and long non-coding RNAs).

41 Results: We observe dysregulation of genes involved in calcium homeostasis, particularly affecting
42 the T-cell activity and regulation, causing disturbed Ca^{2+} induced apoptotic pathway of T cells in
43 IBM muscles. Additionally, LCK/p56, which is an essential gene in regulating the fate of T-cell
44 apoptosis, shows altered expression and splicing usage in IBM muscles

45 Interpretation: Our analysis provides a novel understanding of the molecular mechanisms in IBM by
46 showing a detailed dysregulation of genes involved in calcium homeostasis and its effect on T-cell
47 functioning in IBM muscles. Loss of T-cell regulation is hypothesized to be involved in the consistent
48 observation of no response to immune therapies in IBM patients. Our results show that loss of
49 apoptotic control of cytotoxic T cells could indeed be one component of their abnormal cytolytic
50 activity in IBM muscles.

Introduction

Inclusion body myositis (IBM) is a late-onset, acquired muscle disease with unclear etiology, and the poorly understood molecular pathogenesis is under debate due to several factors. The CD8⁺ T-cell infiltration and overexpression of class I MHC antigens in all muscle fibers indicate an autoimmune cascade and are, in fact, the most consistent finding together with the degeneration of myofibers. However, IBM largely remains refractory to immunosuppressive drugs [1], and comprehensive clinical trials have generally been ineffective [2]. A partial clinical and histopathological overlap with other rimmed-vacuolar (RV) myopathies [3] including accumulations of similar proteins in the RVs [4] support a degenerative pathophysiology. Accumulation/aggregation of these misfolded proteins suggests that IBM could be a protein aggregate disease with immune-mediated cytotoxic inflammation as a resulting secondary feature [5]. However, there is a significant variance in nature and the number of accumulated proteins observed in the IBM muscle biopsies [6]. Similar aggregates observed in HIV-associated IBM [7] suggest that protein aggregation can still be a downstream effect of immune dysfunction. Additionally, the occurrence of rare familial cases [8] and a strong association with immune MHC locus 8.1 ancestral haplotype [9, 10] support a possible genetic predisposition for IBM.

Analysis of tissue-specific mRNAs and subsequent RNA-seq based transcriptomics studies focused on understanding the expression of genes, participating pathways, and networks can increase our understanding of underlying pathomechanisms. Prior studies have investigated the differential gene expression in IBM muscles for both the inflammatory and the degenerative pathology [11-17]. However, no study has attempted a comprehensive analysis of RNA-seq data combining differential gene expression, differential exon, and splicing usage along with an in-depth analysis of the relation between dysregulation of coding and regulatory RNAs in IBM muscles.

Our study used RNA extracted from muscle biopsies of IBM patients, of non-myositis RV-myopathy disease group, and a histopathologically group. We first studied the differential expression of coding, long non-coding RNAs (lncRNAs), and micro RNAs (miRNAs) and then evaluated their possible interplay. Additionally, we studied the transcriptome-wide differential exon and splicing usage. We observed a significant association with genes involved in various calcium-related pathways and identified disturbed calcium regulation specific to T cells in IBM muscles, highlighting the relevance of calcium homeostasis for T-cell activity in IBM muscles. In particular, we identified calcium-induced T lymphocyte apoptosis to be disturbed in IBM muscles.

83 **Materials and methods**

84 Patients and skeletal muscle biopsies

85 Muscle biopsies (predominantly Tibialis anterior or Vastus lateralis) from 24 Finnish patients
86 diagnosed with clinically and pathologically defined IBM according to the ENMC criteria [18] were
87 included. The age of onset was 60 ± 11 years (median $\pm$ SD), and the age at muscle biopsy was $70 \pm$
88 9 years. Additionally, muscle biopsies from six patients with genetically diagnosed Tibial muscular
89 dystrophy (TMD, caused by heterozygous FINmaj mutation the titin gene) [19] were included. In the
90 TMD cohort, the age of onset was 49 ± 11 years, and age at biopsy 54 ± 14 years. Nine muscle
91 biopsies from individuals that underwent leg amputation for reasons other than a muscle disease [20]
92 were also included. These nine biopsies did not show pathologically defined muscle degeneration or
93 inflammation. Age at sampling for amputees was 70 ± 11 years. All muscle biopsies were snap-frozen
94 and stored at -80°C . Muscle biopsies were collected at the Tampere Neuromuscular Research Center,
95 Tampere University Hospital, Finland.

96 RNA extraction, selection, and library preparation

97 Muscle tissue homogenization steps were performed using SpeedMill PLUS (Analytik Jena AG,
98 Germany). RNA was extracted with Qiagen RNeasy Plus Universal Mini Kit (Qiagen, Hilden,
99 Germany) and treated with Invitrogen TURBO DNase buffer (ThermoFisher Scientific, MA, USA)
100 according to the manufacturers' instructions. RNA was quantified and qualitatively assessed using
101 High Sensitivity RNA ScreenTape (Agilent Technologies, CA, USA) on Agilent 4200 TapeStation
102 system (Agilent Technologies).

103 Library preparations and sequencing were performed at Oxford Genomics Center, University of
104 Oxford. For PolyA+ RNA selection, the NEBNext Ultra II Directional RNA Library Prep kit (E7760)
105 for Illumina (NEB, Beverly, MA, USA) was used to prepare strand-specific RNA-seq libraries.
106 Libraries were multiplexed and sequenced on HiSeq4000: 75bp paired-end sequencing (Illumina,
107 CA, USA), and an average of ~ 47 million reads per sample were produced. Samples with enough
108 RNA were used for library preparation for small RNA (< 200 nt) selection (18 IBM, nine amputees,
109 and four TMD). NEBNext Small RNA Library Prep Set (E7330) for Illumina was used per the
110 manufacturer's instructions (NEB). Libraries were multiplexed and sequenced on HiSeq2500: 50bp
111 single-end sequencing (Illumina), and an average of ~ 10 million reads per sample were produced.

112 RNA-seq data pre-processing, QC, and alignment

Adapter sequences and low-quality bases were removed with fastp [21]. Trimmed sequences were then mapped with STAR 2.7.0d [22] (STAR, RRID: SCR_004463) with index generated from Gencode.v29 human reference (release date 05.2018, based on ENSEMBL GRCh38.p12) and comprehensive gene annotation (primary assembly) using the STAR two-pass method according to the guidelines from the ENCODE project for alignment of long RNA (>200 nt) and small RNA (<200 nt) data.

RNA-seq quantification and differential gene expression analysis

Uniquely mapped fragments were summarized and quantified (referred to as counts) by featureCounts [23] (featureCounts, RRID: SCR_012919) using Gencode.v29 primary comprehensive gene annotation, which lists 58,780 RNAs including 19,969 protein-coding, 16,066 non-coding, and 22,745 other types of RNAs (primary gene expression analysis). Separate quantification of counts for lncRNA (lncRNA analysis) was done using long non-coding RNA gene annotation from Gencode.v29 (a subset of the primary annotation). Quantification of counts for miRNAs (miRNA analysis) in 31 samples was done using miRBase human miRNA annotation (Release 22.1 October 2018) [24]. Differential gene expression analysis was performed with DESeq2 [25] (v1.26.0) (DESeq2, RRID: SCR_015687) in Rstudio (v1.2.5019) (RStudio, RRID: SCR_000432) based on R (v3.6.3) (R Project for Statistical Computing, RRID: SCR_001905). Counts were normalized with variance stabilizing transformation function within DESeq2. A principal component analysis (PCA) was performed on the gene expression data of the IBM samples compared to amputee and TMD groups. Further, pairwise comparisons between cohorts were performed using the Wald test. Log₂ fold changes (LFC) were shrunk using 'ashr' adaptive shrinkage estimation [26], and results were generated with default independent filtering for increasing power. Only genes with LFC values larger than ± 1.5 and a Benjamini-Hochberg adjusted p-value of ≤ 0.01 were considered further. Genes specifically dysregulated in IBM muscles were considered for downstream analysis.

Pathway analysis

Ingenuity Pathway Analysis (IPA, QIAGEN Inc.) (Ingenuity Pathway Analysis, RRID: SCR_008653) was used for pathway analysis and enrichment analysis of the obtained differential gene expression data. Using Ingenuity Pathways Knowledge Base (Ingenuity Pathways Knowledge Base, RRID: SCR_008117), IPA mapped and annotated genes to the pathways and predicted activation state based on the direction of changes comparing it with the change in the database.

143

144 Differential splicing analysis

145 To investigate differential usage of exons and splicing, independent of the differential gene
 146 expression analysis, we used QoRTS [27] java-based application (v1.3.6) (QoRTs, RRID:
 147 SCR_018665) to prepare counts from exons and splice junctions (known and novel) from the aligned
 148 data. Downstream analysis of this data was performed using JunctionSeq [28] (v1.16.0) in R.
 149 JunctionSeq results produce a q-value (based on FDR) on gene-level analysis, which considers that
 150 one or more exon/junction in this gene is differentially used. A conservative q-value threshold of 0.01
 151 was used to select significant observations. IBM-specific differentially expressed genes and
 152 differentially spliced genes were compared (Fig. 1). Statistical over-enrichment analysis for Gene
 153 ontology terms in categories: Molecular function, biological process, and cellular component, was
 154 performed on results obtained from QoRTs/JunctionSeq using clusterProfiler [29] (clusterProfiler,
 155 RRID: SCR_016884). Gene sets were compared using UpSet plot [30].

156 **Results**

157 Expression signature in IBM muscles

158 Fig. 1a shows the summarized workflow of the methodology. The PCA shown in Fig. 1b explains the
 159 differences between the three cohorts. Pairwise comparisons were performed to reduce the potential
 160 confounding effects of groups, which identified 2,288 and 302 genes specifically up- or down-
 161 regulated in the IBM cohort, respectively (Fig. 1c). Non-coding RNA analyses resulted in 497
 162 lncRNAs upregulated, 106 lncRNAs downregulated, 140 miRNAs upregulated, and 126 miRNAs
 163 explicitly downregulated in the IBM cohort compared to other groups. These IBM-specific
 164 dysregulated RNAs were used for downstream pathway analysis using IPA workflow. The top 15
 165 genes dysregulated specifically in IBM muscles, with their functional annotations and normalized
 166 expression in the different cohorts, are shown in Fig. 2.

167 Pathway analysis

168 We performed IPA workflow analysis on IBM-specific dysregulated genes to better understand the
 169 pathways and the upstream regulators associated with the observed expression dysregulation. Out of
 170 these, 2,588 genes, 596 lncRNAs, and 257 miRNAs mapped to the Ingenuity database. From the
 171 primary gene expression analysis, IPA identified 91 pathways as significantly altered. Table 1 shows
 172 a summary of the IPA results with the top identified pathways.

173 The top upstream regulators in both miRNA and lncRNA analysis are shown in table 2 and table 3,
 174 respectively. We identified an increased expression of the lncRNA *DNM3OS* (DNM3 antisense RNA)

175 and *MIAT* (Myocardial infarction associated transcript) from these analyses. IPA suggested this
176 dysregulation may be due to *JDP2* (Jun Dimerization Protein 2) and *TARDBP* (TAR DNA Binding
177 Protein), acting as an upstream regulator of *DNM3OS* and *MIAT* respectively (table 4).

178 Dysregulation of calcium-related pathways in IBM muscles

179 IPA identified calcium-induced T lymphocyte apoptosis as one of the most significant pathways
180 dysregulated in IBM muscles (table 1). Our IBM-specific dataset contained 69 genes with significant
181 dysregulation out of the 232 genes annotated in this pathway. A part of this pathway, including the
182 major players, is shown in Fig. 3. Another pathway outside the top results identified that 29 genes
183 (29/208, $p = 7.05E-03$) significantly dysregulated in our dataset are also involved in calcium
184 signaling. These results prompted us to investigate further for calcium related issues in cellular
185 signaling, and we found that IPA also detects dysregulation of the following processes, mobilization
186 of Ca^{2+} (80 genes), the release of Ca^{2+} (33 genes), quantity of Ca^{2+} (51 genes) and flux of Ca^{2+} (51
187 genes), as significantly disturbed in IBM muscles (table 4).

188 Altered exon usage and splicing pattern in IBM muscles

189 To explore IBM-specific exon usage, we performed an independent transcriptome-wide differential
190 splicing analysis in our three cohorts. We obtained a list of 1,271 differentially spliced genes in IBM
191 from our differential splicing analysis. These transcripts either showed IBM-specific increased usage
192 of a known junction or a known exon or contained a novel exon-exon junction resulting in an
193 alternative isoform. To understand the diverse portfolio of mature mRNAs created from pre-mRNAs,
194 we used gene ontology over-enrichment analysis on these 1,271 differentially spliced genes and
195 identified the first splicing signature specific to IBM muscles. To understand the different classes
196 over-represented in these genes, we performed statistical over-enrichment analysis using
197 clusterProfiler for all three GO categories as seen in Fig. 4 a,b,c. Our analysis showed an enrichment
198 of genes involved in the structure and organization of actin filaments assembly in IBM muscles and,
199 interestingly, proteins involved in mRNA processing and metabolism.

200 We then compared the list of differentially spliced genes with differentially expressed genes in our
201 analysis and found an overlap of 79 genes (Fig. 1d). Next, we wanted to observe the overlap between
202 six different sets of genes, namely IBM specific differentially spliced genes, calcium-induced T
203 Lymphocyte apoptosis, Mobilization of Ca^{2+} , Flux of Ca^{2+} , Quantity of Ca^{2+} , and Release of Ca^{2+}
204 (Fig. 4d). We observed 10 genes to be associated with calcium-related processes; *HLA-DPA1*, *HLA-*
205 *DPB1*, and *HLA-DQB1* are associated with calcium-induced T Lymphocyte apoptosis, *ANXA1* is
206 associated with mobilization, flux, and release of Ca^{2+} , *CCL4* is associated with mobilization, flux,

207 and quantity of Ca^{2+} , *GRK3* and *RARRES2* are associated with mobilization, *SH3KBP1* with flux, and
208 *ITGAM* with the quantity of Ca^{2+} . In particular, one specific differentially spliced gene, *LCK*, is part
209 of all six sets.

210 Fig. 5a shows the gene expression of *LCK* in three cohorts, with expression in IBM muscles being
211 significantly higher than the others ($\log_2\text{FC} = +2.86$, $\text{padj}=3.50\text{E-}11$, $\text{ranking} = 355/2590$).
212 Additionally, Fig. 5b shows the differential splicing pattern observed in *LCK* in all three groups. The
213 highlighted E016 corresponds to an alternative exon (chr1:32274818-32274992, GRCh38).

214 Discussion

215 In this study, we aimed to identify a more detailed IBM-specific molecular signature, using different
216 RNA-seq based methods that can help us explore the inflammatory and degenerative parts in depth.
217 Antigen-driven T-cell cytotoxicity is the most reproducible and plausible part of the complex
218 molecular pathomechanism in IBM. However, it remains unknown what antigen drives this IBM-
219 specific immune cascade.

220 As part of the RV pathology, accumulated proteins or the unfolded protein response have been
221 hypothesized to prompt an immune reaction [5]. A recent unbiased proteomics study dissected these
222 RVs in IBM [31]. Interestingly, the protein encoded by one of our top differentially expressed genes,
223 *MYL4*, is also detected in the RVs in IBM along with *ANXA1*, which is both differentially expressed
224 and differentially spliced in IBM muscles. In our study design, we considered TMD, another RV
225 muscle disease but without immune involvement, to understand if there are any RV-specific antigens
226 in IBM muscles. Additionally, using age-matched histopathologically normal muscles from
227 amputees, we aimed to understand if general inflammatory signatures can be replicated and studied
228 in more detail using additional methods such as non-coding RNAs and differential splicing studies.
229 Consequently, our strong study design and robust methodology helped us replicate findings from
230 previous studies [11-17] and identify essentially new calcium-related issues in IBM muscles and their
231 link with the altered T-cell cytotoxicity in IBM muscle fibers.

232 We found that several genes contributing to calcium homeostasis are differentially expressed in IBM
233 muscles resulting in dysregulation of several critical pathways, specifically, calcium-induced T
234 lymphocyte apoptosis and related Nur77 signaling. Ca^{2+} is a universal second messenger in T cells,
235 and it is known to regulate proliferation and differentiation of T cells and T-cell effector functions
236 [32]. The complexity and duration of Ca^{2+} signals and resultant cytoskeletal rearrangements
237 determine the fate of T cells in response to an antigen [33]. On one hand, a short-term increase in
238 intracellular Ca^{2+} concentration results in the cytolytic activity of T cells; on the other hand, prolonged

elevation results in proliferation, differentiation, and maturation of naïve T cells into Th1, Th2, and Th17 subtypes and the production of cytokines[32].

Ca²⁺ signaling is known to optimize the interaction between T cells and antigen-presenting cells [33]. The binding of antigen/MHC complexes (CD8⁺-MHC class I/CD4⁺-MHC class II) to T-cell receptors (TCR) activates Src-family protein tyrosine kinases, e.g., LCK and FYN at the cytoplasmic side of the TCR/CD3 complex. Additionally, activation of ZAP-70, a tyrosine kinase associate protein, results in the phosphorylation and activation of the intracellular enzyme phospholipase C-γ1 (PLC-γ1) [32, 33]. PLC-γ1 hydrolyses phosphatidylinositol 4,5-biphosphate (PIP2) to produce two other second messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptor (IP3R) on the endoplasmic reticulum (ER) membrane to promote rapid release of Ca²⁺ from ER to the cytosol [32]. However, this release of Ca²⁺ is insufficient for antigen-derived T-cell fate but results in depletion of intracellular Ca²⁺ triggering a rapid influx of Ca²⁺ through activation and opening of Ca²⁺ release-Ca²⁺ activated channels (CRAC) on the plasma membrane formed by different STIM1/ORAI1 combinations [34]. The duration of Ca²⁺ influx is vital for activating the calcineurin-dependent nuclear factor activate T cells (NFAT) transcription pathway [32]. In the cytoplasm, calcineurin removes excess phosphate residues from the N terminus of NFAT, promoting its entry into the nucleus. Disruptions in NFAT signaling can cause several phenotypes, including cardiovascular, musculoskeletal, and immunological diseases [35]. Meanwhile, DAG, another secondary messenger, activates protein kinase C (PKC), which in turn activates the nuclear factor kappa B (NFκB). The duration and complexity of Ca²⁺ signals drive the NFAT/NFκB signaling and determine downstream T-cell activation.

The genes in the NR4A family (NR4A1/Nur77, NR4A2/Nurr1, NR4A3/Nor1) act as critical molecular switches in cell survival and inflammation. Human *NR4A1* encodes for a homolog of a mouse protein called Nur77, a zinc transcription factor expressed as an early gene in T cells upon antigen-TCR interaction. In addition to being a transcriptional activator, Nur77 has an apoptotic role in T regulatory fate [36] and other non-genomic proapoptotic functions via mitochondrial interactions with Bcl-2 [37]. T cells deficient in Nur77 have been shown to have high proliferation, enhanced T-cell activation, and increased susceptibility for T-cell-mediated inflammatory diseases [38]. The expression of Nur77 is Ca²⁺ dependent and is controlled by the myocyte enhancer factor 2 (MEF2) transcription factor [39], whose DNA-binding and transcriptional activity is enhanced by Calcineurin. Another calcium-dependent transcription factor, CABIN1, acts as a transcriptional repressor of MEF2, thus keeping the Nur77 promoter silent in the absence of a TCR signal [40]. The interaction between CABIN1 and calcineurin is influenced by intracellular Ca²⁺ and PKC activation, resulting in

hyperphosphorylation of CABIN1 and its subsequent transcription repressing activity. An increase in intracellular Ca^{2+} concentration activates the interaction of the calmodulin family of genes (CALM) with CABIN1, triggering the dissociation of MEF2 from Cabin and MEF2 to become transcriptionally active [41]. In the nucleus, NFAT interacts with MEF2 and enhances its transcriptional activity by recruiting the co-activator p300 for the transcription of Nur77.

In our dataset, 69 genes mapping to the calcium-induced T Lymphocyte apoptosis and 72 genes mapping the Nur77 signaling in T Lymphocytes are differentially expressed in IBM muscles. As seen in Fig. 3, several essential genes like *ZAP70*, *LCK*, different subunits of Protein Kinase C, and *ATP2A1* which encodes for SERCA, are significantly changed in IBM muscles. Additionally, we also observed genes associated with the mobilization of Ca^{2+} , the release of Ca^{2+} , the quantity of Ca^{2+} , and the flux of Ca^{2+} as significantly dysregulated in IBM muscles, indicating a possible widespread disturbance with the handling of calcium entry and release in cells. In T cells, especially, this disturbance could dramatically impact their activation, differentiation, and most likely, the regulation of T-cell apoptosis will be disturbed.

Apoptosis in T cells is necessary to resolve their inflammatory activity, and defective or delayed apoptosis may contribute to the pathogenesis of inflammatory diseases [42]. In this scenario, loss of apoptotic control could be one mechanism explaining the lack of immune-suppressive therapeutic effect in IBM [43].

The diversity of the skeletal muscle proteome is, among others, dependent on the diversity of exon usage in pre-mRNAs [44]. From our transcriptome-wide splicing analysis within the differentially expressed genes, we identified 79 genes, out of which ten are associated with different calcium-related functions. Amongst these, *LCK* is a T lymphocyte-specific protein tyrosine kinase involved in downstream events of antigen-TCR interaction. *LCK*/p56 is essential in transducing signals leading to apoptotic cell death in mature T cells [45], and its activity is tightly regulated to protect against hyperactivation of T cells and autoimmunity, thus maintaining T-cell homeostasis [46]. Moreover, *LCK* also selectively influences the flux and release of calcium in cells [47]. In our analysis, *LCK* is both differentially expressed and differentially spliced in IBM muscles. Disturbed T-cell apoptosis and the dysregulation of *LCK* in IBM muscles provide novel insights into the molecular mechanisms of IBM. Considering the crucial regulatory activity of *LCK*, it might be a potential therapeutic target for IBM patients.

We also observe dysregulation of several non-coding RNAs in our study. Previously, Hamann and colleagues have discussed lncRNAs in the context of IBM [13]. The benefits of our study design,

304 especially the homogenous molecular pathology and the larger sample size, let us dig deeper into the
 305 dysregulation of lncRNAs specific to IBM muscles. We identified that JDP2 (DNA binding
 306 transcription factor) and TARDBP/TDP-43 (DNA and RNA binding protein) might have altered
 307 regulator activity since their downstream non-coding partners (*DNM3OS* and *MIAT*, respectively) are
 308 significantly overexpressed IBM muscles. Additionally, both these proteins are specific to RNA
 309 polymerase II (RNA Pol II), facilitating transcription and pre-mRNA maturation. Alteration in RNA
 310 or DNA binding proteins (expression or localization) associated with the activity of the spliceosome
 311 machinery can directly affect the downstream events. Since TDP-43 is accumulated in RVs, one
 312 possibility is that the unavailability of TDP-43 can affect its transcription and splicing activities. The
 313 normal expression of *TARDBP* we observe in IBM patients is expected and is in coherence with the
 314 previous reports [48]. In inherited muscle diseases, damaging variants in the disease-associated gene
 315 can result in mislocalization and accumulation of mutant protein in the muscle fibers. Previous studies
 316 have reported rare exonic variants in genes, including *VCP* and *SQSTM1* in IBM [49, 50]. However,
 317 in our cohort of IBM patients, there were no rare exonic *TARDBP*, *VCP*, or *SQSTM1* variants [9] that
 318 could suggest a possible association with abnormal protein turnover and accumulation/aggregation.
 319 Therefore, further evidence to suggest the pathogenic role of variants in such genes and their
 320 downstream effect on pathological protein accumulation in IBM is still missing. However, the
 321 potential downside of TDP-43 not being available for its traditional roles, such as effective splicing,
 322 because of the aggregation is noteworthy. Further evidence of possible dysregulation of splicing in
 323 IBM muscles comes from our differential splicing results where proteins involved in mRNA
 324 processing, transcription, and regulation are enriched, suggesting that additional studies are needed
 325 to understand the possible impact of dysregulated mRNA processing in IBM muscles.

326 Previously, Pinal-Fernandez and colleagues observed that calcium-induced T lymphocyte apoptosis
 327 was a significant IBM-specific dysregulated pathway in their extensive analysis of different
 328 inflammatory myopathies but did not comment further on the possible importance [17]. Additionally,
 329 using a smaller sample size, Amici and colleagues identified calcium signaling as one of the
 330 significant disturbed pathways in IBM muscles and hypothesized its potential major role [14].
 331 Furthermore, previous gene expression studies in IBM have analyzed data primarily from microarrays
 332 [11, 12, 15]. Only recently paired-end reads RNA-seq have been used in IBM studies [13, 14, 16, 17].
 333 While microarray-based analyses are comparable for differential expression studies, RNA-seq based
 334 methodologies are superior for in-depth transcriptome analysis. In our study design, we used matched
 335 muscle biopsies and state-of-the-art RNA-seq analysis tools. Our analyses show novel molecular
 336 events in IBM muscles which increase our understanding of IBM and provide valuable additions to

improve the therapeutic interventions considering the disturbed calcium homeostasis, dysregulation of LCK, and associated deregulation of apoptotic control of T cells in IBM muscles.

Acknowledgments

The authors would like to thank the patients and their families. We thank the Oxford Genomics Centre at the Wellcome Centre for Human Genetics (funded by Wellcome Trust grant reference 203141/Z/16/Z) for the generation and initial processing of the sequencing data. We would like to thank CSC – IT Center for Science, Finland, for its computational resources. We thank Merja Soininen, Talha Qureshi and Eini Penkkimäki for their technical assistance. We would like to thank Peter-Bram 't Hoen for his critical review of this manuscript.

Declarations

Funding

This work was supported by the Folkhälsan Research Foundation, Doctoral program in Integrative Life Science (ILS) and Doctoral school in Health Sciences (DSHealth), University of Helsinki (MJ), the Päivikki and Sakari Sohlberg Foundation (MJ), the Biomedicum Helsinki Foundation (MJ), Finska läkaresällskapet (BU/MJ), the Finnish Medical Foundation (JP), the Paulo foundation (MS), the Jane and Aatos Erkko Foundation (PH) and the Sigrid Jusélius Foundation (BU).

Author Contributions

MJ: Conceptualization of the study, funding acquisition, data analysis and curation, methodology, project administration, visualization, writing the original draft, review, and editing of the manuscript.

AV: data analysis, methodology, writing the original draft.

JP: Patient samples and data collection, writing the original draft.

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MS: Conceptualization of the study and methodology, review and editing of the manuscript.

PH: Funding acquisition, project administration, and review and editing of the manuscript.

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367 Conflicts of interest

368 The authors report no conflicts of interest.

369 Ethical approval

370 The study was performed in line with the principles of the Declaration of Helsinki. Ethical approval
371 for this study falls under HUS:195/13/03/00/11. Informed consent from the patients was obtained at
372 the time of sample collection.

373 Data availability

374 Raw counts and normalized DESeq2 counts from polyA⁺ RNAs and miRNAs are available in GEO
375 as superseries GSE151758.

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550

551 Figures legends

552 **Fig. 1** a) Workflow and methodology used in this study. b) Principal component analysis of gene
553 expression results showing the pairwise comparison between different groups: IBM, TMD and
554 Amputees. c) IBM-specific differentially expressed genes were determined by comparing IBM cases
555 with amputee and TMD groups. d) Comparison between IBM specific differentially expressed genes
556 (cyan) and IBM specific differentially spliced genes (magenta).

557

558 **Fig. 2** a) Top 15 differentially expressed genes specific to IBM muscles. Log₂ fold change (log2FC)
559 of IBM versus amputees calculated by DEseq2 after shrinkage estimations. '+'/'-' sign denotes the
560 direction of change, i.e., positive log2FC values indicate overexpressed genes in IBM muscles, and
561 negative log2FC values indicate underexpressed genes in IBM muscles. The p-value of significance
562 and adjusted p-value using the Benjamini-Hochberg corrections and associated GO terms are shown
563 for each gene. Genes marked with * are also observed as significantly dysregulated in Hamann et al.
564 [13] b) Normalized gene expression in the different cohorts is presented as boxplots. Median and
565 quartile values are shown, with whiskers reaching up to 1.5 times the interquartile range. Individual
566 expression levels are shown as jitter points. The raincloud plots illustrate the distribution of data in
567 each cohort. The scaled Y-axis shows log normalized counts.

568

569 **Fig. 3** The calcium-induced T lymphocyte apoptosis pathway with gene expression changes observed
570 in IBM compared to groups. Created with BioRender.com

571

572 **Fig. 4** Statistical over-representation tests were performed on a list of differentially spliced RNAs,
573 using clusterProfiler for a) Biological Processes, b) Cellular component, and c) Molecular function.
574 d) An UpSet plot is shown comparing six different sets, namely, IBM specific differentially spliced
575 (1,271 genes), mobilization of Ca²⁺ (80 genes), calcium-induced T lymphocyte apoptosis (69 genes),
576 the flux of Ca²⁺ (51 genes), quantity of Ca²⁺ (51 genes), and release of Ca²⁺ (33 genes). Dots and lines
577 represent subsets of different lists. The horizontal bar graph (wine color) represents the size of each
578 set, while the vertical histogram (black) represents the number of RNAs in each subset. The 10 RNAs
579 that are both differentially expressed and differentially spliced are shown with a red circle with their
580 gene names (black).

581

582 **Fig. 5** a) Normalized LCK expression in the different cohorts (as explained in Fig. 2b). b) Altered
583 isoform expression of *LCK* using JunctionSeq showing estimated normalized mean read-pair count
584 for each exon and splice junctions in the different cohorts (left) as well as for the whole *LCK* gene
585 (right). The significantly alternatively spliced feature, E016 (pink), corresponds to chr1:32274818-
586 32274992 (GRCh38). The alternative *LCK* transcripts used in the JunctionSeq analysis are shown
587 below with their corresponding ENSEMBL identifiers.

588

Tables

Table 1: Top 10 dysregulated canonical pathways identified by IPA. The significance of the identified pathway is shown with a p-value and the number of differentially expressed genes observed in the IBM-specific dataset compared to the number of genes present in the database for each pathway.

Ingenuity Canonical Pathways	p-value	Number of genes in dataset / Number of genes in database
Dendritic Cell Maturation	5.72E-31	106/357
T Cell Receptor Signaling	2.30E-25	97/355
T Cell Exhaustion Signaling Pathway	3.12E-25	94/338
Cdc42 Signaling	7.63E-24	88/315
iCOS-iCOSL Signaling in T Helper Cells	4.10E-23	81/280
CD28 Signaling in T Helper Cells	1.13E-22	82/290
OX40 Signaling Pathway	4.03E-21	68/222
Calcium-induced T Lymphocyte Apoptosis	1.29E-20	69/232
Nur77 Signaling in T Lymphocytes	3.07E-20	72/253
Role of NFAT in Regulation of the Immune Response	4.57E-19	87/360

Table 2: From the miRNA analysis, upstream binding partners are shown along with their target miRNA. A p-value and associated GO terms are shown.

Upstream regulator	p-value	GO terms and annotations
<i>AGO2</i>	7.89E-23	RNA polymerase II complex binding
<i>SSB</i>	4.74E-19	RNA binding
<i>TP53</i>	7.59E-09	Transcription regulatory region sequence-specific DNA binding
RNA polymerase III	4.09E-06	Synthesis of small RNA, RNA polymerase activity

Table 3: From the long non-coding RNA analysis, upstream binding partners are shown along with their target lncRNA. A p-value and associated GO terms are shown

Upstream regulator	Target molecule in dataset	p-value	GO terms and annotations
JDP2	<i>DNM3OS</i>	4.10E-03	DNA-binding transcription factor activity, RNA polymerase II-specific
miR-338-3p	<i>NR2F1-AS1</i>	4.10E-03	Negative regulation of gene expression; negative regulation of IL-6 production; negative regulation of cytokine production involved in inflammatory response
miR-150-5p	<i>MIAT</i>	4.10E-03	mRNA binding involved in posttranscriptional gene silencing
TARDBP	<i>MIAT</i>	2.03E-02	RNA polymerase II cis-regulatory region sequence-specific DNA binding
mir-150	<i>MIAT</i>	2.63E-02	mRNA binding involved in posttranscriptional gene silencing
PGF	<i>DNM3OS</i>	3.43E-02	Protein binding, signal transduction
FUS	<i>RMRP</i>	3.43E-02	mRNA binding, mRNA stabilization
DDX58	<i>EGOT</i>	4.61E-02	double-stranded RNA binding

602

Table 4: In cell signaling processes, different pathways associated with calcium homeostasis are shown along with their p-value and a prediction state.

Functional annotations	p-value	Predicted activation state
Mobilization of Ca²⁺	5.04E-26	Increased
Flux of Ca²⁺	4.68E-13	Increased
Quantity of Ca²⁺	4.34E-09	Increased
Release of Ca²⁺	5.00E-09	Increased

605

a

b

c

d

a

Symbol	Mean count	log2FC	pvalue	padj	GO terms
<i>KDM7A</i>	1147.06	-1.69	4.07E-32	2.39E-28	Iron ion binding and oxidoreductase activity
<i>LGALS3BP*</i>	2922.28	2.59	5.91E-31	2.89E-27	Scavenger receptor activity
<i>TLR3</i>	128.42	2.29	3.98E-28	1.30E-24	Regulation of dendritic cell cytokine production
<i>ATP6V0A1</i>	3393.79	-1.76	3.01E-27	8.83E-24	ATPase binding and proton-transporting ATPase activity
<i>IRF8*</i>	199.90	3.89	4.66E-27	1.24E-23	DNA-binding transcription factor activity, RNA polymerase II-specific
<i>SLC7A7</i>	216.91	3.16	2.08E-26	5.09E-23	Amino acid transmembrane transporter activity
<i>FGL2</i>	1102.10	3.27	3.14E-26	6.58E-23	T cell activation via T cell receptor contact with antigen bound to MHC molecule on antigen presenting cell
<i>B2M*</i>	46381.30	2.29	4.71E-26	9.22E-23	Positive regulation of T cell mediated cytotoxicity
<i>MYL4*</i>	610.22	5.44	5.92E-26	1.09E-22	Actin monomer binding, Calcium ion binding
<i>UBE2L6*</i>	986.03	2.47	1.86E-25	3.04E-22	Ubiquitin-protein transferase activity
<i>SPPL2B</i>	1161.90	-1.66	2.99E-25	4.39E-22	Protein homodimerization activity
<i>TUBA1A*</i>	2951.15	2.50	4.36E-25	5.81E-22	GTPase activity, structural molecule activity
<i>TFAP4</i>	234.41	-1.52	4.32E-25	5.81E-22	Transcription regulatory region sequence-specific DNA binding
<i>HLA-DRA</i>	8658.66	3.25	7.29E-25	9.00E-22	Antigen processing and presentation of endogenous peptide antigen via MHC class II
<i>SOX11</i>	96.02	4.03	1.97E-24	2.23E-21	DNA-binding transcription factor activity, RNA polymerase II-specific

b

a

b

c

d

a

b