Depressed β-adrenergic inotropic responsiveness and intracellular calcium handling abnormalities in Duchenne Muscular Dystrophy patients’ induced pluripotent stem cell–derived cardiomyocytes

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Abstract
Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene, is an X-linked disease affecting male and rarely adult heterozygous females, resulting in death by the late 20s to early 30s. Previous studies reported depressed left ventricular function in DMD patients which may result from deranged intracellular Ca\(^{2+}\)-handling. To decipher the mechanism(s) underlying the depressed LV function, we tested the hypothesis that iPSC-CMs generated from DMD patients feature blunted positive inotropic response to β-adrenergic stimulation. To test the hypothesis, [Ca\(^{2+}\)]\(_i\) transients and contractions were recorded from healthy and DMD-CMs. While in healthy CMs (HC) isoproterenol caused a prominent positive inotropic effect, DMD-CMs displayed a blunted inotropic response. Next, we tested the functionality of the sarcoplasmic reticulum (SR) by measuring caffeine-induced Ca\(^{2+}\) release. In contrast to HC, DMD-CMs exhibited reduced caffeine-induced Ca\(^{2+}\) signal amplitude and recovery time. In support of the depleted SR Ca\(^{2+}\) stores hypothesis, in DMD-CMs the negative inotropic effects of ryanodine and cyclopiazonic acid were smaller than in HC. RNA-seq analyses demonstrated that in DMD CMs the RNA-expression levels of specific subunits of the L-type calcium channel, the β1-adrenergic receptor (ADRB1) and adenylate cyclase were down-regulated by 3.5-, 2.8- and 3-fold, respectively, which collectively contribute to the depressed β-adrenergic responsiveness.

Keywords
[Ca\(^{2+}\)]\(_i\) transients, contractions, duchenne muscular dystrophy, induced pluripotent stem cell–derived cardiomyocytes, RNA-sequencing, SR Ca\(^{2+}\) stores, β-adrenergic responsiveness
Duchenne muscular dystrophy (DMD), the most common of 9 types of muscular dystrophy, is an X-linked disease affecting boys and teenagers and rarely adult heterozygous females. The incidence in male newborns is 1:3500, and the prevalence is 6:100 000 in the male population. The disease is caused by mutations in the dystrophin gene encoding the dystrophin protein and constitutes the most severe childhood form of the broader family of muscular dystrophies. Dystrophin is a key structural-functional protein providing strength and stability to the contracting muscle and is essential for maintaining healthy muscle function; its lack leads to cell damage, impaired Ca\(^{2+}\) homeostasis, elevated oxidative stress and reduced energy production in muscle cells. Consequently, loss of sarcolemmal dystrophin and dystrophin-glycoprotein complex (DGC) promotes muscle fibre damage during muscle contraction. Symptoms onset usually occurs between ages 3 and 5, and includes progressive muscle weakness and wasting. By the late teens, the heart and respiratory muscles are also affected, ultimately leading to death due to respiratory and/or cardiac failure.

Dilated cardiomyopathy (DCM), which is a key pathological feature in DMD patients, affects nearly all patients and is a major cause of morbidity and mortality. Accordingly, many studies reported depressed left ventricular contractile function in DMD patients which may result from impaired β-adrenergic signalling cascade and/or intracellular Ca\(^{2+}\)-handling. The latter is supported by: (a) our recent study in DMD induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) demonstrating prominent arrhythmias in the form of delayed afterdepolarizations (DADs) known to result from Ca\(^{2+}\)-overload; (b) studies in mdx mice cardiomyocytes showing ‘leaky’ Ryanodine receptor (RyR) and abnormal Ca\(^{2+}\) handling. To decipher the mechanism(s) underlying the depressed ventricular function in DMD patients, we tested the hypothesis that DMD patients’ iPSC-CMs feature blunted positive inotropic response to β-adrenergic stimulation. Our major findings are: (a) the inotropic responses to isoproterenol and elevated [Ca\(^{2+}\)]\(_i\) are blunted in DMD cardiomyocytes; (b) defective β-adrenergic receptor cascade is not responsible for the blunted inotropic response in DMD cardiomyocytes; (c) sarcoplasmic reticulum (SR) Ca\(^{2+}\)-handling machinery is altered in DMD cardiomyocytes; (d) depressed SR Ca\(^{2+}\) release in DMD cardiomyocytes is likely to underlie the attenuated positive inotropic response; and (e) RNA-expression levels of specific subunits of the L-type calcium channel, the β1-adrenergic receptor (ADRβ1) and adenylate cyclase are down-regulated in DMD cardiomyocytes by 3.5- to 2.8- and 3-fold, respectively, which may collectively contribute to the depressed β-adrenergic responsiveness. These novel findings provide an explanation for the depressed ventricular function in DMD patients and thus may help to introduce therapeutic support for the patients.

**2 | METHODS**

**2.1 | Tissue collection from DMD patients**

Dermal biopsies were obtained from a 50-year-old DMD female manifesting carrier with a deletion of exons 8-12 (ex.8_12del) and a 32-year-old DMD male patient carrying a substitution of cytosine to thymine (c.5899C>T) constituting a premature stop codon. The donors signed a consent form according to approval #7603-09-SMC by the Helsinki Committee for Experiments on Human Subjects at Sheba Medical Center, Ramat Gan, Israel. iPSCs generation and characterization, karyotype analysis, teratoma formation, genotyping and pluripotency evaluation by immunofluorescence staining and flow cytometry were previously described. Differentiation into cardiomyocytes was performed according to the directed differentiation by modulating Wnt/β-catenin signalling.

**2.2 | Measurements of intracellular Ca\(^{2+}\) transients, contractions and action potentials**

All methods are detailed in Supplementary Material. In brief, intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) transients and contractions were measured from iPSC-CMs composing embryoid bodies (EBs), using the IonOptix Calcium and Contractility system (Westwood, MA, USA), as previously described. Transmembrane action potentials were recorded by means of the whole cell patch clamp.

**2.3 | Western blot**

See details in Supplementary Material.

**2.4 | RNA extraction and RNA-seq analysis**

RNA was extracted from control and DMD cardiomyocytes using the ‘ReliaPrep’ RNA Cell Miniprep System Kit (Promega, Fitchburg, WI, USA). RNA-seq libraries were produced and the RNA-seq data were analysed. See details in Supplementary Material. The raw RNA-Seq data were deposited and released in the SRA database, with the BioProject accession number PRJNA688142.

**2.5 | Statistical analysis**

Results are presented as mean ± SEM. See details in Supplementary Material.

**3 | RESULTS**

**3.1 | DMD iPSC-CMs exhibit blunted β-adrenergic inotropic responsiveness**

All control experiments were performed on healthy male and female (clones fse5m and 24.5) cardiomyocytes. The first step was to determine whether the two healthy clones have similar [Ca\(^{2+}\)]\(_i\)-handling and contractile machineries. As seen Figure S1, the [Ca\(^{2+}\)]\(_i\) transient and contraction parameters are similar in the two healthy
clones (fse5m and 24.5), and therefore, one lumped healthy (control) group was used for comparison with adult male and adult female DMD cardiomyocytes. In support of our hypothesis, adult male and adult female DMD cardiomyocytes displayed a blunted positive inotropic response to isoproterenol compared to healthy cells (Figure 1). Specifically, except for [Ca$^{2+}$] transient amplitude in adult male, all other [Ca$^{2+}$] transient parameters in adult male and adult female were augmented to a smaller extent by isoproterenol, compared to healthy cells. A similar blunted response in adult male and adult female cardiomyocytes was observed respecting the contraction parameters; contraction amplitude (Lamp), maximal rate of contraction (+dL/dt) and maximal rate of relaxation (−dL/dt) (Figure 1G-I). The extrapolation of these results to DMD patients sheds light on the mechanisms underlying their depressed ventricular function.

### 3.2 Are the [Ca$^{2+}$]$_i$-handling and contractile machineries depressed in DMD cardiomyocytes?

To determine whether the blunted β-adrenergic inotropic response in DMD cardiomyocyte is due to depressed [Ca$^{2+}$]$_i$-handling and contractile machineries we compared [Ca$^{2+}$] transient and contraction parameters in healthy, adult male and adult female iPSC-CMs. As illustrated in Figure S2, [Ca$^{2+}$] transient amplitude and all the contraction parameters were comparable in DMD and healthy cardiomyocytes. The exceptions were: (a) maximal rate of [Ca$^{2+}$] rise in adult male was smaller than in healthy cardiomyocytes (Figure S2B); (b) maximal rate of [Ca$^{2+}$] rise in adult female was larger than healthy and adult male cardiomyocytes (Figure S2B); (c) maximal rate of [Ca$^{2+}$] decay in adult male was larger than healthy cardiomyocytes (Figure S2C); (d) maximal rate of [Ca$^{2+}$] decay in adult female was...
smaller than adult male cardiomyocytes (Figure S2C). Collectively, these findings show that the \([\text{Ca}^{2+}]_i\) transients and contractions are not impaired in DMD cardiomyocytes, indicating that the blunted \(\beta\) -adrenergic inotropic response did not result from depressed \([\text{Ca}^{2+}]_i\) handling and contractile machineries.

3.3 | Is the \(\beta\)-adrenergic signalling cascade impaired in DMD cardiomyocytes?

To determine whether the blunted inotropic response was caused by impaired \(\beta\)-adrenergic signalling cascade in DMD cardiomyocytes, we measured the positive chronotropic response to isoproterenol. As we previously reported\(^{16}\) and shown here as well (Figure 2A,B), DMD cardiomyocytes exhibit lower \((P < 0.001\) and \(P < 0.01\) in adult male and adult female, respectively) spontaneous firing rate compared to healthy cardiomyocytes. In support of the functionality of the \(\beta\)-adrenergic signalling cascade, adult male and adult female cardiomyocytes presented a healthy-like positive chronotropic response to isoproterenol. Further, adult male cardiomyocytes exhibited a larger chronotropic response than healthy cells (Figure 2C), which is in agreement with the increased heart rate in DMD patients.\(^{24}\) From these experiments we concluded that an impaired \(\beta\)-adrenergic cascade is not responsible for the blunted positive inotropic response in DMD cardiomyocytes.

3.4 | Intracellular \(\text{Ca}^{2+}\) handling machinery is altered in DMD cardiomyocytes

Since we excluded the possibility that the blunted inotropic response is due to depressed \([\text{Ca}^{2+}]_i\)-handling and contractile machineries, we tested the hypothesis that impaired downstream element(s) mediating positive inotropic interventions is depressed in DMD cardiomyocytes.

3.4.1 | The ionotropic effect of elevated \([\text{Ca}^{2+}]_o\)

Here, we tested the inotropic response to a non-receptor-mediated positive inotropic intervention, elevated \([\text{Ca}^{2+}]_o\) (2, 3, 4 and 5 mmol/L), which operates by augmenting the L-type Ca\(^{2+}\)
current ($I_{Ca,L}$), which in turn increases SR Ca$^{2+}$ release and contractile force. As seen in Figure 3A, while in healthy cardiomyocytes, elevating [Ca$^{2+}$]$_o$ caused a positive inotropic effect, DMD cardiomyocytes were hardly affected (Figure 3B-C). Specifically, except for [Ca$^{2+}$] transient amplitude, the response of all other [Ca$^{2+}$] transient and contraction parameters to elevated [Ca$^{2+}$]$_o$ was blunted in DMD compared to healthy cardiomyocytes (Figure 3D-I).

### 3.4.2 SR Ca$^{2+}$ handling

Because the inotropic response to isoproterenol and elevated [Ca$^{2+}$]$_o$ was blunted in DMD cardiomyocytes, we hypothesized that this was due to a downstream common element—depleted SR Ca$^{2+}$ stores. This hypothesis was tested by investigating: (a) caffeine-induced RyR-mediated SR Ca$^{2+}$ release; (b) the inotropic effects of ryanodine and cyclopiazonic acid (CPA), both interfering with SR Ca$^{2+}$-handling. 26,27

#### 3.4.3 Caffeine-induced RyR-mediated SR Ca$^{2+}$ release

RyR-mediated SR Ca$^{2+}$ release was measured by a brief application of caffeine (10 mmol/L), a RyR2 opener. 28 As illustrated in Figure 4, DMD and healthy cardiomyocytes differed in their response to caffeine. As we previously reported, 21,22,29 in healthy cardiomyocytes caffeine caused an abrupt increase in [Ca$^{2+}$]i, associated with contraction cessation, followed by a decline in [Ca$^{2+}$]i, along with resumption of contractions after ~25 seconds (Figure 4A). In contrast, the response of adult male and adult female cardiomyocytes was shorter and smaller (Figure 4B-D). The caffeine response was quantified by calculating 3 parameters: (a) recovery time—the time from the

**FIGURE 3** The effects of different concentrations of [Ca$^{2+}$]$_o$ on the [Ca$^{2+}$]$_i$ transients and contractions in healthy, DMD adult male and DMD adult female iPSC-CMs. (A-C) Representative [Ca$^{2+}$]$_i$ transients and contractions from healthy, adult male and adult female iPSC-CMs in the presence of different concentrations of [Ca$^{2+}$]$_o$. (D) [Ca$^{2+}$]$_i$ transient amplitude (Ramp). (E) Maximal rate of [Ca$^{2+}$]$_i$ rise (+d[Ca$^{2+}$]/dt). (F) Maximal rate of [Ca$^{2+}$]$_i$ decay (−d[Ca$^{2+}$]/dt). (G) Maximal amplitude (Lamp). (H) Maximal contraction rate (+dL/dt). (I) Maximal relaxation rate (−dL/dt). iPSC-CMs were stimulated at a frequency 20% higher than the spontaneous firing rate. The effect of different concentrations of [Ca$^{2+}$]$_o$ on the [Ca$^{2+}$]$_i$ transient parameters of adult male (n=11), adult female (n=15) and healthy, n = 22) cardiomyocytes was expressed as percent change of Control (2 mmol/L [Ca$^{2+}$]$_o$). The effect of different concentrations of [Ca$^{2+}$]$_o$ on contraction parameters of adult male (n=10), adult female (n=17) and healthy (n=17) cardiomyocytes was expressed as percent change of Control (2 mmol/L [Ca$^{2+}$]$_o$). *P < 0.05, **P < 0.01, ***P < 0.001 (vs Control, 2 mmol/L [Ca$^{2+}$]$_o$). Two-way ANOVA test was performed followed by Holm-Sidak posthoc test.
peak of caffeine-induced \([\text{Ca}^{2+}]_i\), rise to the first \([\text{Ca}^{2+}]_i\) transient; (b) the per cent change in caffeine-induced \([\text{Ca}^{2+}]_i\) signal amplitude compared to pre-caffeine amplitude; (c) the per cent change in area of the caffeine-induced \([\text{Ca}^{2+}]_i\) signal compared to the pre-caffeine area; (d) the mean recovery time, calculated as the time from the peak of caffeine-induced \([\text{Ca}^{2+}]_i\), rise to the first measurable \([\text{Ca}^{2+}]_i\) transient. Adult male \((n = 16)\) iPSC-CMs; adult female \((n = 8)\) iPSC-CMs; healthy \((n = 16)\) iPSC-CMs. *\(P < 0.05\), ***\(P < 0.001\). Asterisks above bars connecting columns represent significant difference between groups. One-way ANOVA test was followed by Holm-Sidak Posthoc test

3.4.4 | The negative inotropic effects of ryanodine and cyclopiazonic acid (CPA) on DMD iPSC-CMs

If SR \(\text{Ca}^{2+}\) stores are depleted in DMD cardiomyocytes, ryanodine \((30 \mu\text{mol/L}, \text{Ref. 30})\) is expected to have a smaller inhibitory effect than in healthy cells. Indeed, as depicted by the representative contraction recordings and the summary, the contraction amplitude was decreased to a lesser \((P < 0.01, P < 0.05)\) extent in adult male and adult female respectively, than in healthy cardiomyocytes (Figure 5A-D). To further support the DMD depleted SR \(\text{Ca}^{2+}\) stores hypothesis, we investigated the negative inotropic effect of CPA, a SERCA inhibitor.27 Like ryanodine, the contraction amplitude was less reduced in adult male and adult female than in healthy cardiomyocytes (Figure 5E-H).

3.5 | The molecular mechanisms underlying the blunted positive inotropic response in DMD cardiomyocytes

To decipher the molecular mechanisms underlying the blunted positive inotropic response in DMD cardiomyocytes, we carried out a comprehensive RNA-seq analysis, comparing healthy and DMD cardiomyocytes. Altogether, the analysis (see Methods)
detected 19,389 genes, of which we focused on 119 genes that were included in the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for (cardiac) intracellular calcium signalling, contraction and adrenergic signalling. Briefly, KEGG is a database resource for understanding high-level functions of biological systems, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular data sets generated by genome sequencing and other high-throughput experimental technologies. Accordingly, all 119 genes included in the 3 KEGG pathways are illustrated in the combined waterfall plot (Figure 6). The differently expressed genes (DEGs) are aligned according to the degree of change of the expression level, measured by fold change in DMD cardiomyocytes compared to healthy control. For the sake of clarity, the waterfall was split into a left panel and a right panel, including down-regulated and up-regulated genes, respectively. Significant changes in expression levels are indicated by colour. Bright colours indicate significant changes at the False Discovery Rate (FDR)-corrected P-value, and milder colours indicate significance according to nominal P-value. In an attempt to explain the functional findings described above, based on the KEGG and the corresponding waterfall data, we generated a map (Figure 7) which depicts the major elements of the intracellular calcium signalling, contraction and adrenergic signalling, albeit the extrapolation from transcriptomic data to function is not straightforward. Notably, there are many more genes in the waterfall plot than boxes in the map, since many of the boxes refer to multimeric complexes or to a class of receptors, including some referencing to over a dozen different genes, such as DHPR, PP2A and GPCR. The first functional finding supported by the RNA-seq data is that the $[\text{Ca}^{2+}]_i$-handling and contractile machineries properties are comparable in healthy and DMD cardiomyocytes (Figure S2). Specifically, key elements of these machineries—RyR2 (type 2 Ryanodine receptor), SERCA2 (Sarcoplasmic/Endoplasmic Reticulum type 2), calsequestrin (CASQ2), phospholamban (PLN), troponins and actin—are all similar in both...
groups. As depicted in Figure 7, several alternations in gene expression were detected in discrete components constituting the myosin light chain complex. Specifically, whereas myosin light chain kinase (MYLK) and MYLK2 are down-regulated by ~6-fold in DMD cardiomyocytes, myosin light chain 2 (MYL2) is up-regulated by 2-fold. Further, we found down-regulation by 3.5-fold of genes that encode proteins of specific subunits of the L-type calcium channel, CACNA2D2 and CACNG6, encoding for alpha-2/delta subunit and gamma subunit, respectively. Finally, two genes that encode the plasma membrane Ca\textsuperscript{2+}-pump are differentially regulated in DMD cardiomyocytes; ATP2B3 is down-regulated while ATP2B2 is up-regulated. The next key functional finding for which we searched for support in the RNA-seq data is the depressed β-adrenergic inotropic response in DMD cardiomyocytes. Indeed, the expression of the β1-adrenergic receptor (ADRβ1) which predominantly mediates the positive inotropic effect isoproterenol in the heart\textsuperscript{31-33}; is down-regulated in DMD cardiomyocytes by 2.8-fold, compared to healthy cells. In contrast, the expression of β2-adrenergic receptor (ADRβ2) which accounts for ~20% of all cardiac β-adrenoreceptors,\textsuperscript{33} and whose activation increases contractile force and heart rate, is not altered in DMD cardiomyocytes. A major finding clearly contributing to the depressed β-adrenergic inotropic response, is the >3-fold decrease in the expression levels of the key enzyme adenylate cyclase (AC) isoforms III and V (ADCY3, ADCY5)\textsuperscript{34,35} in DMD cardiomyocytes. Briefly, G-protein mediated activation of AC, increases cAMP, activating protein kinase A (PKA) which in turn phosphorylates several targets, such as phospholamban, LTCC and Troponin I.\textsuperscript{36,37}

3.6 | Western blot analysis of ATP2ase2 SERCA2 in DMD iPSC-CMs and healthy iPSC-CMs

To further understand the molecular mechanisms underlying the depressed inotropic response of DMD cardiomyocytes and the depleted SR Ca\textsuperscript{2+} stores, we performed Western blot analysis of ATPase 2 SERCA2 in healthy and DMD iPSC-CMs (Figures S3 and S4). As shown in Figures S3 and S4, compared to healthy, SERCA2 is overexpressed in adult male and adult female cardiomyocytes. Based on this finding, we suggest that impaired [Ca\textsuperscript{2+}]\textsubscript{i} handling may be compensated by overexpression of SERCA2 in DMD iPSC-CMs.

4 | DISCUSSION

To decipher the mechanisms underlying the depressed ventricular function in DMD patients we tested the hypothesis that DMD iPSC-CMs feature blunted positive inotropic response to
β-adrenergic stimulation. The major findings were: (a) blunted positive inotropic response to isoproterenol and elevated \([\text{Ca}^{2+}]_o\); (b) healthy-like positive chronotropic response in DMD cardiomyocytes; (c) depressed SR \([\text{Ca}^{2+}]_o\) release in DMD cardiomyocytes, likely to underlie the attenuated positive inotropic response; (d) RNA-expression levels of specific subunits of the L-type calcium channel, β₁-adrenergic receptor (ADRβ₁) and adenylate cyclase are down-regulated in DMD cardiomyocytes, which collectively may contribute to the depressed β-adrenergic inotropic responsiveness.

4.1 | The β-adrenergic positive inotropic response is blunted in DMD cardiomyocytes

Since a clinical hallmark of DMD cardiomyopathy is depressed ventricular function, we attempted to determine the cellular basis of this major pathology. Indeed, we found that male and female DMD cardiomyocytes exhibit a blunted positive inotropic response to β-adrenergic stimulation (Figure 1) which can account for the depressed cardiac function in DMD patient. To decipher the mechanisms underlying the blunted response, we tested whether the following elements are impaired in DMD cardiomyocytes: (a) β₁-adrenergic signalling cascade; (b) the \([\text{Ca}^{2+}]_o\)-handling capacity; (c) SR \([\text{Ca}^{2+}]_o\) release in DMD cardiomyocytes, which collectively suggest that indeed in DMD cardiomyocytes SR- \([\text{Ca}^{2+}]_o\) release and contractile machineries are similar in healthy and DMD cardiomyocytes, despite the dystrophin mutations, can be explained by the experimental conditions in which the cardiomyocytes are unloaded, and thus not subjected to mechanical stress which is expected to cause depressed contractile function in DMD cardiomyocytes.

4.1.1 | Is the β-adrenergic receptor impaired in DMD cardiomyocytes?

A depressed β-adrenergic inotropic response can result from aberrant function and/or decreased receptor density in DMD cardiomyocytes. This option was negated by the electrophysiological results showing comparable and increased response of DMD adult female and adult male cardiomyocytes, respectively, to isoproterenol, compared to healthy cells (Figure 2). Collectively, these findings suggest that in DMD cardiomyocytes the β-adrenergic receptor is functional, and even hyperactive (in male cardiomyocytes), which may contribute to the higher heart rate in DMD patient. To further support the notion of defective SR function in DMD cardiomyocytes, we tested the effect of ryanodine which blocks \([\text{Ca}^{2+}]_o\)-induced SR- \([\text{Ca}^{2+}]_o\) release. In agreement with studies in iPSC-CMs, hESC-CMs and rabbit cardiomyocytes, in healthy cardiomyocytes ryanodine (30 μmol/L) attenuated all contraction parameters. As predicted from \([\text{Ca}^{2+}]_o\)-depleted SR stores, in DMD cardiomyocytes the negative inotropic response was smaller than in healthy cardiomyocytes (Figure 5). To strengthen the hypothesis of \([\text{Ca}^{2+}]_o\)-depleted SR \([\text{Ca}^{2+}]_o\) stores in DMD iPSC-CMs, we compared the negative inotropic effect of the SERCA2 inhibitor CPA, in healthy and DMD iPSC-CMs. Like ryanodine, in DMD cardiomyocytes CPA caused a smaller inotropic effect than in healthy cardiomyocytes (Figure S5), suggesting that SERCA2 function in DMD differs from healthy cardiomyocytes.
Further, the overexpression of SERCA in DMD cardiomyocytes can account for a small negative inotropic effect of CPA in DMD cardiomyocytes.

The results from the caffeine, ryanodine and CPA experiments suggest that in DMD cardiomyocytes, the depressed positive inotropic response to isoproterenol and elevated \([\text{Ca}^{2+}]_o\) result in part from depleted SR \(\text{Ca}^{2+}\) stores; a most likely cause underlying this phenomenon is leaky RyR2. In this regards, Fauconnier et al. reported that structural and functional remodelling of the cardiac SR \(\text{Ca}^{2+}\) release channel/ryanodine receptor (RyR2) occurs in cardiomyocytes from \(\text{mdx}\) mice. RyR2 from \(\text{mdx}\) hearts were S-nitrosylated and depleted of calstabin2 (FKBP12.6), resulting in 'leaky' RyR2 channels and a diastolic SR \(\text{Ca}^{2+}\) leak. This report on 'leaky' RyR2 channels from \(\text{mdx}\) hearts supports our proposed mechanism that in DMD cardiomyocytes the SR is \(\text{Ca}^{2+}\) depleted.

4.1.5 | The molecular mechanisms underlying the blunted positive inotropic response in DMD cardiomyocytes

To decipher the molecular mechanisms contributing to the blunted positive inotropic response in DMD cardiomyocytes, we performed a comprehensive RNA-seq analysis. Importantly, we found that the expression of the \(\beta_1\)-adrenergic receptor (ADR\(\beta_1\)), predominantly expressed in the heart was down-regulated by 2.8-fold in DMD compared to healthy cardiomyocytes. The next step downstream to ADR\(\beta_1\) is activation of adenyl cyclase (AC) via Gs, resulting in increased cAMP levels. The primary target of cAMP is protein kinase A (PKA) which phosphorylates several key proteins essential for cardiac function, such as the L-type calcium channel, phospholamban, troponin I and RyR. Therefore, because ADR\(\beta_1\) is down-regulated, its stimulation will result in attenuated AC activation, decreased cAMP levels, and consequently blunted positive inotropic response to isoproterenol. Our finding that ADR\(\beta_1\) expression is decreased in DMD cardiomyocytes is supported by Li et al. who showed that the abundance of ADR\(\beta_1\) protein is reduced by 2/3 in 4-month-old \(\text{mdx}\) hearts compared to control hearts. Additionally, Lu et al examined \(\beta_1\)-adrenergic receptors from both left and right atria in young (12 week) and old \(\text{mdx}\) male mice (12 month), compared with their age- and sex-matched healthy controls (C57). Briefly, \(\text{mdx}\) mice exhibited decreased response to isoprenaline compared to control mice. In addition, young and old \(\text{mdx}\) mice expressed a reduction in calcium-induced positive inotropy compared to control mice, implying calcium handling abnormalities in \(\text{mdx}\) cardiac muscle. Overall, the complex alterations in \(\beta_1\)-adrenergic mechanisms and the dysfunction of calcium homeostasis may both contribute to the abnormalities of contractile function in \(\text{mdx}\) myocardium.

The comprehensive RNA-seq analysis further show that the AC III and V isoforms are down-regulated by 3-fold in DMD...
cardiomyocytes. Specifically, AC V which is the major cardiac isoform regulates heart rate and contractility. Therefore, down-regulation of AC, especially type V, contributes to reduced cAMP levels and to depressed β-adrenergic responsiveness. Another important element involved in the β-adrenergic inotropic responsiveness is the L-type Ca\(^{2+}\) channel responsible for Ca\(^{2+}\) entry during the plateau of the action potential, thereby initiating contraction. The RNA-seq analysis show a 3.5-fold down-regulation of the genes CACNA2D2\(^{50}\) and CACNG6 encoding for alpha-2/delta and gamma subunits,\(^{51}\) respectively of the L-type Ca\(^{2+}\) channel. This finding is supported by Viola et al\(^{49}\) who demonstrated that Ca\(^{2+}\) influx is increased in mdx cardiomyocytes, as a consequence of delayed activation of I\(_{Ca,L}\) thereby contributing to increased cytoplasmic Ca\(^{2+}\). Moreover, activation of I\(_{Ca,L}\) was shown to increase mitochondrial Ca\(^{2+}\), NADH and superoxide concentrations, as a result of increased uptake of Ca\(^{2+}\) into the mitochondria. Therefore, the L-type Ca\(^{2+}\) channel contributes to impairments in mitochondrial Ca\(^{2+}\) handling in mdx ventricular cardiomyocytes. Although RNA levels of the L-type Ca\(^{2+}\) channel were down-regulated, the [Ca\(^{2+}\)]\(_{i}\) transient properties in DMD cardiomyocytes did not differ from healthy cells. This may imply that the decrease in CACNA2D2 and CACNG6 expression was not significant enough to cause functional changes at a basal resting state of the channel. However, under stress conditions such as β-adrenergic stimulation, the lack of sufficient channel expression manifested in DMD iPSC-CMs contributes to the blunted β-adrenergic inotropic responsiveness. Finally, MYL2 was found to be up-regulated by 2 fold in DMD cardiomyocytes. MYL2, known as the regulatory light chain of myosin, is a sarcomeric protein that plays a role in heart development and function. Following phosphorylation, MYL2 is involved in cross-bridge cycling kinetics and cardiac muscle contraction.\(^{52,53}\) This finding is consistent with Baker et al\(^{54}\) who demonstrated by means of RT-PCR that MYL2 transcription is up-regulated in mice lacking both dystrophin and utrophin as well as in mdx myotubes. The up-regulation of MYL2 in mdx myotubes strengthens our finding in DMD cardiomyocytes.

### 4.1.6 | The SERCA data

To further decipher putative mechanisms underlying the depleted SR Ca\(^{2+}\) stores in DMD iPSC-CMs, we found that phosphorylated SERCA2 is overexpressed by 11-fold in adult male and by 2.75 in adult female compared to healthy cardiomyocytes (Figures S3 and S4). This finding is consistent with similar findings in mdx mice, which express increased levels of the slow Ca\(^{2+}\)-pump isoform (SERCA2a) (2-fold) in skeletal muscle.\(^{55}\) In contrast to the protein expression findings, RNA-seq analysis did not show SERCA overexpression, which may indicate that while the overall transcription levels are not changed, SERCA2 activity is increased, as demonstrated by the overexpression of SERCA2 which probably underwent posttranslational modifications. Increased SERCA activity was previously demonstrated to have a beneficial effect on failing hearts,\(^{52,53,56-58}\) and therefore, the increase SERCA expression can constitute a compensatory attempt to increase SR Ca\(^{2+}\)-stores and subsequently DMD iPSC-CMs inotropy.

### 5 | SUMMARY AND CONCLUSIONS

Our results demonstrate that DMD iPSC-CMs exhibit blunted positive inotropic response to isoproterenol and elevated [Ca\(^{2+}\)]\(_{o}\) compared to healthy cardiomyocytes. In addition, the results from the caffeine, ryanodine and CPA experiments suggest that in DMD cardiomyocytes these depressed responses result from depleted SR Ca\(^{2+}\) stores. We propose that SERCA2 overexpression in DMD cardiomyocytes constitutes a compensatory mechanism to increase SR Ca\(^{2+}\)-stores and subsequently DMD iPSC-CMs inotropy. The RNA-seq data demonstrate that down-regulation of ADRβ1 and major AC isoforms contribute to the blunted positive inotropic response to isoproterenol. These results emphasize the involvement of abnormal β-adrenergic cascade and [Ca\(^{2+}\)]\(_{i}\)-handling in DMD cardiac pathophysiology and may provide the basis for further research and the development of new therapeutic agents targeting these abnormalities.

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### CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

### AUTHOR CONTRIBUTIONS

Lucy N. Mekies: Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing-original draft (lead); Writing-review & editing (lead). Danielle Regev: Formal analysis (supporting); Methodology (equal). Binyamin Eisen: Data curation (supporting); Formal analysis (supporting); Writing-review & editing (supporting); Methodology (equal). Jonatan Fernandez-Gracia: Formal analysis (supporting); Methodology (equal). Polina Baskin: Writing-review & editing (supporting); Methodology (equal). Ronen Ben Jehuda: Investigation (supporting); Methodology (supporting). Rita Shulman: Methodology (supporting). Irina Reiter: Methodology (supporting). Raz Palty: Conceptualization (supporting); Investigation (supporting). Michael Arad: Conceptualization (supporting); Investigation (supporting). Eyal Gottlieb: Conceptualization (supporting); Resources (supporting). Ofer Binah: Conceptualization (lead); Resources (lead); Supervision (lead); Validation (lead); Visualization (lead).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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