# Indicators of Apoptosis in Duchenne Muscular Dystrophy Patients

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## ABSTRACT

**Background:** Tissue sections of dystrophic muscle demonstrate apoptotic myonuclei in degenerating muscle fibers of Duchene muscle dystrophy (DMD) patients. The apoptosis cascade can be triggered by 2 main pathways, via an intrinsic, endogenous system such as the mitochondrial Bax/Bcl-2 or via an extrinsic system involving transmembrane receptors of the death receptor family Fas and Fas Ligand (FasL).
**Aim of the Work:** The present study is an attempt to demonstrate the levels of Fas and FasL and Bax/Bcl-2 in DMD pathogenesis.
**Patient and Methods:** Subjects were 16 boys with DMD diagnosed clinically and at the molecular level versus 20 age and socioeconomic matching healthy boys.
**Results:** Plasma DNA fragmentation (0.38%±0.12 vs. 0.2%±.0.1.5) and Fas (9.9±2.8 vs. 2±0.1, $p<0.001$) together with FasL mRNA expression in circulating lymphocytes (0.47±.09 vs. 0.24±.04, $p<0.001$) were significantly increased in DMD patients compared to controls. There was a significant increase in Bax mRNA relative concentration (0.19±0.07 vs. 0.05±0.01, $p<0.00001$) accompanied by a significant decrease in Bcl-2 protein in circulating lymphocytes (6.4±1.6 vs10±2.8, $p<0.00001$) both compared to controls.
**Conclusion:** Indicate that apoptosis and its markers determined in blood of DMD patients can replace the invasive technique of tissue biopsy.

**Key Words:**

Duchenne muscular Dystrophy, apoptosis, Bax, Bcl-2, Fas, FasL

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## INTRODUCTION

Duchenne muscular dystrophy is an X-linked recessive disorder, primarily characterized by progressive muscle weakness and waisting. The disease results from the absence of dystrophin.[1] Although, the precise molecular mechanisms leading to muscle pathology are poorly understood, dystrophic muscles

undergo increased oxidative stress and altered calcium homeostasis, which may contribute to myofiber loss by triggering both necrosis and apoptosis.[2] In humans, DNA-fragmentation and expression of apoptosis-related proteins indicate that apoptosis plays a role in muscle degeneration and regeneration in muscular dystrophies.[3]

The apoptosis cascade can be triggered by 2 main pathways, via an intrinsic, endogenous system such as the mitochondrial Bax/Bcl-2 or via an extrinsic system Fas nd FasL involving transmembrane receptors of the death receptor family.[4] Fas ligand is a 40-kDa type II membrane protein that belongs to the tumor necrosis factor superfamily and induces apoptosis through cognate interaction with its receptor Fas.[5] In contrast to Fas, which is expressed ubiquitously, FasL is mainly present in activated T lymphocytes, natural killer cells, and macrophages[5] Activation of the Fas receptor through interaction with transmembrane FasL leads to the recruitment and activation of numerous signaling molecules, including apoptosis and inducing transcription factors. This mechanism is crucial for the suicide of activated T cells and macrophages.[6,7] However this mechanism has not been previously measured in blood of DMD patients.

The aim of the present study is to measure apotosis and markers of inflammatory cell induced apoptosis in blood of DMD patients.

## PATIENTS AND MPETHODS

Patients were 16 boys with DMD diagnosed clinically and at the molecular level versus 20 age and socioeconomic matching healthy boys. Patients and controls were chosen to be, free from any infection and receiving no therapeutic treatment known to increase the oxidative stress. Blood samples were drawn after their parents' consent.

### Methods

Reverse Transcriptase - polymerase Chain Reaction (RT-PCR) Analysis for FAS-Ligand and Bax.

Total RNA was extracted from lymphocytes QIAGEN RNA extraction Kit extraction kit (QIAGEN Inc, USA). The RNA samples were reverse transcribed using superscript reverse transcriptase, using QIAGEN OneStep RT-PCR kit (QIAGEN Inc USA, Clini Lab). FasL primer sequences, forward: 5'-CAA GTC CAA CTC AAG GTC CAT GCC-3'; reverse: 5'-CAG AGA GAG CTC AGA TAC GTT- TGAC-3'). Primers for β-actin were synthesized simultaneously as an internal reference for all samples (Forward: 5'-GTG GGG CGC CCC AGG CAC CA-3'; reverse: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'). Bax sequences[8], forward: 5'-CAC CAG CTC TGA- GCA GAT G-3'; reverse: 5'-GCG AGG CGG TGA- GCA CTC C-3'). 5 µl of RT reaction of each of the formed cDNA were processed for PCR. Ten µL from each PCR reaction product were sepa-

rated on a 2% agarose gel then stained with ethidium bromide.

The appearance of specific bands (Bax 516 bp, β-actin 540 and FasL 345 bp) was evaluated under ultraviolet light and photographed.[9]

***Determination of Soluble Fas*** was duplicate in duplicate plasma samples. Soluble Fas protein was measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits.This kit uses the competitive binding enzyme immunoassay format.[10]

***DNA Fragmentation Assay:***
This is done according to the method of Ioannou and Chen.[11] In apoptosis DNA is broken down into fragments ranging from 20-200 base pairs. Separation of both fragmented and total DNA carried out using DNA sperating kit. DNA fragments were gradiently separated from the intact DNA using polyethelene glycol (5% in Ethyl ether) and then quantified spectrophotometrically using Hoechst 33258 (0.2 μg/ml) as a chromophore.

**ELISA Bcl-2:** The amounts of Bcl2 in circulating lymphocytes were determined by a sandwich enzyme linked immunosorbent assay (ELISA) purchased from Cliniulab, using two anti-human BCL2 monoclonal murine antibodies.

**Statistical Analysis** Each experimental condition was performed in triplicate. Data are expressed as mean±SD. Comparisons were made by Student's t-test.

## RESULTS

Percentage of DNA fragmentation per total DNA in plasma showed a significant increase among DMD patients compared to total controls (Mean=0.38%±0.12 vs. 0.2%±.0.1.5, $p<0.001$) as shown in (Table 1) and (Figure 4).

Fas in plasma, as shown in table (1) and (Figure 5), showed a significant increase among DMD patients compared to total controls (Mean 9.9±2.8 vs. 2±0.1, $p<0.001$).

FasL mRNA relative expression (Figure 1) as compared to β-actin mRNA expression (Figure 2) in circulating lymphocytes showed a significant increase among DMD patients compared to total controls (Mean 0.47±.09 vs. 0.24±.04, $p<0.001$) table (1) and (Figure 6).

Bax mRNA relative expression (Figure 3) in circulating lymphocytes as compared to compared to β-actin mRNA expression figure (2) showed a significant increase among DMD patients compared to controls (Mean 0.19±0.07 vs 0.05±0.01, $p<0.001$), (Table 2) and (Figure 7).

Bcl-2 in circulating lymphocytes, as shown in table (2) and figure (7) showed a significant decrease among DMD patients compared to controls (Mean 6.4±1.6 vs. 10±2.8, $p<0.001$).

**Table 1:** FasL, Fas and Percentage of DNA Fragmentation in Blood of DMD Patients Compared to Controls.

| | Percentage DNA Fragmentation | Fas | FasL |
|---|---|---|---|
| DMD patients | 0.38%±0.12 | 9.9±2.8 | 0.47±.09 |
| Controls | 0.2%±.0.1.5 | 2±0.1 | 0.24±.04 |
| t | 3.7 | 17 | 4.2 |
| p | p<0.001 | <0.00005 | <0.005 |

**Table 2:** Bax mRNA and Bcl-2 protein in Blood of DMD Patients Compared to Controls.

| | Bcl-2 protein | Bax mRNA |
|---|---|---|
| **DMD** | 6.4 ± 1.6 | 0.19 ± 0.07 |
| **Controls** | 10 ± 2.8 | 0.05 ± 0.01 |
| | p< 0.001 | p < 0.0000001 |

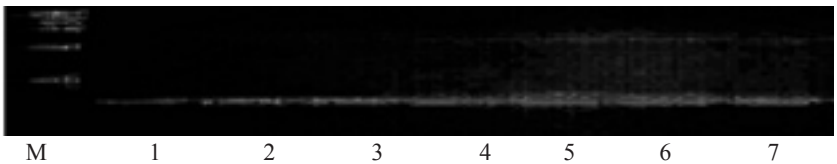


**Fig. 1:** FasL mRNA expression in DMD patients compared to controls.
M denotes marker, lanes 1-3 are controls and lanes 4-7 are DMD patients.

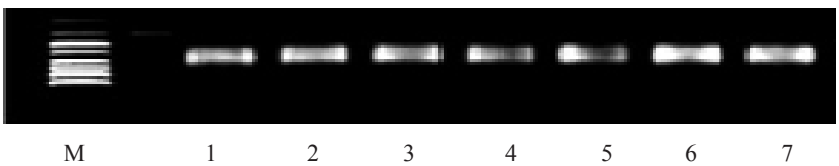


**Fig. 2:** β-actin mRNA expression in DMD patients compared to controls.
M denotes marker, lanes 1-3 are controls and lanes 4-7 are DMD patients.

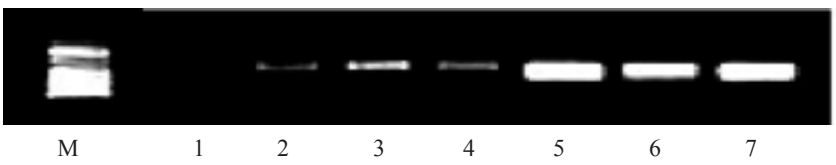


**Fig. 3:** Bax mRNA expression in DMD patients compared to controls.
Lanes 1-3 are controls and lanes 4-6 are DMD patients.

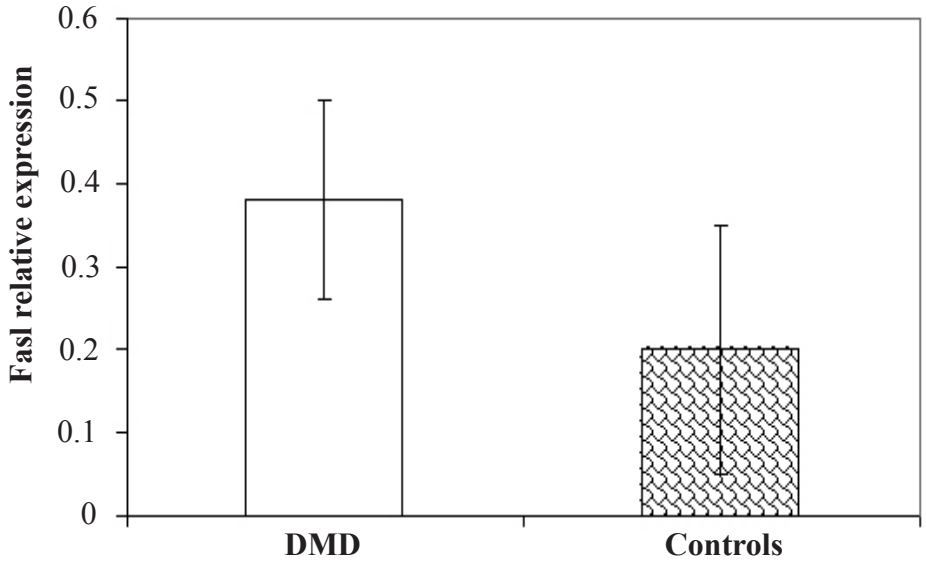


**Fig. 4:** Percentage of DNA fragmentation per total DNA in plasma of DMD patients compared to controls.

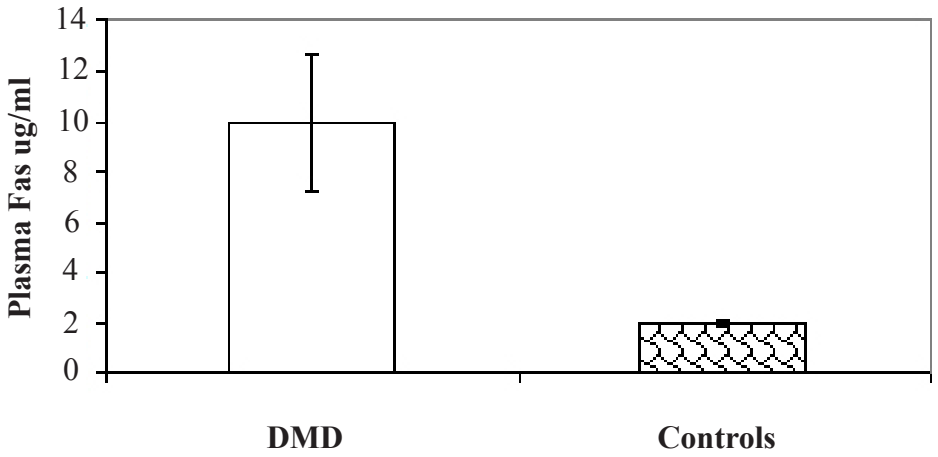


**Fig. 5:** Percentage of DNA fragmentation per total **DNA** in plasma of DMD patients compared to controls.

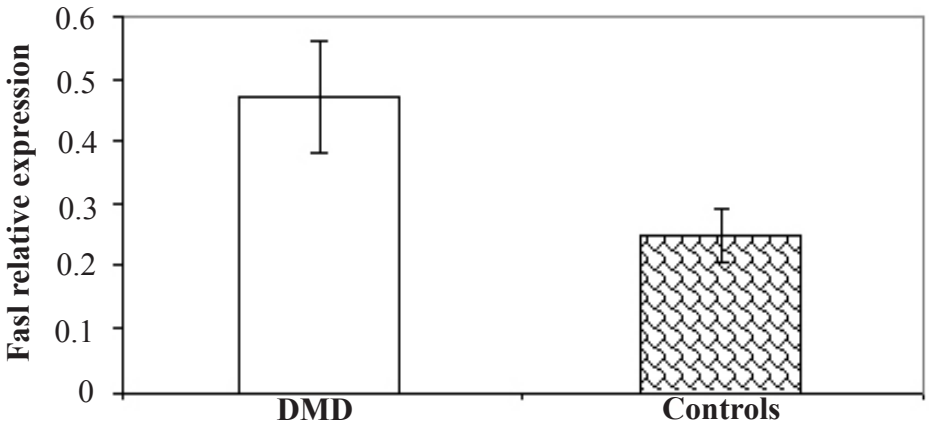


**Fig. 6 :** FasL in circulating lymphocytes of DMD patients compared to controls.

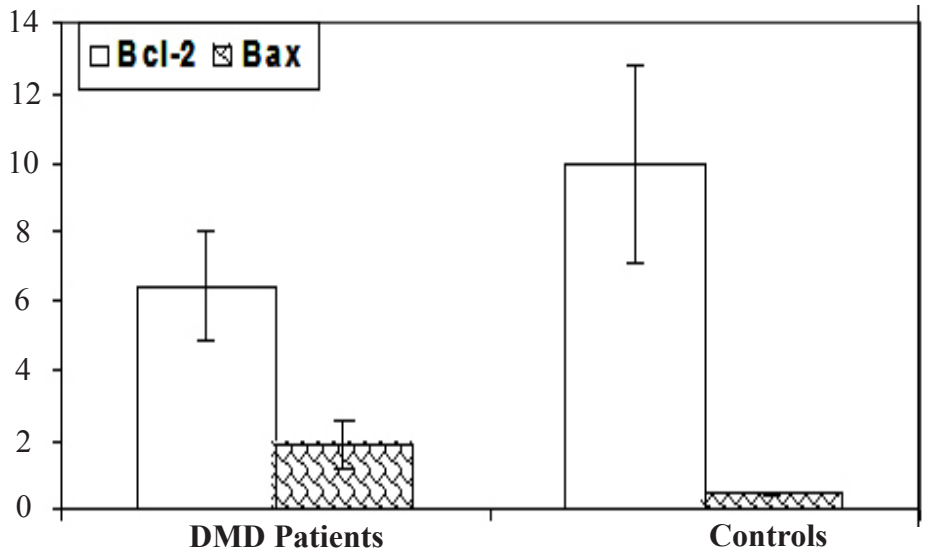


**Fig. 7:** Bax mRNA and Bcl-2 protein in blood of DMD patients vompared to controls.

## DISCUSSION

Dystrophin is thought to play a structural role in providing a link between intracellular actin and extracellular laminin via its interaction with a complex of peripheral and integral membrane proteins, called 'the dystrophin–glycoprotein complex' (DGC).[12] Disruption of this linkage results in membrane instability and dystrophic muscle fibers are highly susceptible to contraction-induced injury[13]. Besides providing mechanical stability, several proteins of the DGC play a role in cell signaling.[14] Altered cell signaling is thought to increase the susceptibility of muscle fibers to secondary triggers, such as functional ischemia and oxidative stress.[15]

Fas is expressed rather ubiquitously in a wide range of normal tissues including muscle cells, while FasL is expressed on activated T cells. Fas/Fas ligand interaction is an important trigger for apoptosis in many cell types expressing Fas as a surface marker.[6] In the present study plasma Fas has been shown to be significantly elevated in DMD patients compared to controls. Increased expression of death factor Fas and decreased expression of the anti-apoptotic protein were shown to be expressed in muscles of DMD patients compared to controls.[16] Fas/FasL is involved in muscle cell apoptosis in at least two of the inflammatory myopathies, in polymyositis (PM), inclusion body myositis (IBM).[17]

The present study showed a significant increase in FasL among DMD patients compared to controls. Previous studiues showed that in inflammatory myopathies, an infiltrate of mononuclear cells capable of synthesizing interferons was present in biopsies from DMD patients. The predominant cell types detected in both diseases were macrophages and T lymphocytes, these two cell types comprising more than 80% of the infiltrating mononuclear cells.[18] FasL are cytotoxic molecules used by CD8+T lymphocytes and natural killer (NK) cells to induce apoptosis.[17] Constitutively expressed by NK cells, perforin, granzyme B, and FasL expression is induced upon activation by antigen presenting cells, such as dendritic cells, in CD8+T lymphocytes.[17]

In the present study a significant increase in plasma DNA fragmentation is also observed in DMD patients compared to controls. Elimination of genetically damaged cells by apoptosis represent an adaptive response mechanism that reduce the risk of cancer and other genetically induced diseases from exposure to radiation or other DNA damaging agents.[19] Muscle exercise-induced apoptosis is a normal regulatory process that serves to remove certain damaged cells without a pronounced inflammatory response, thus ensuring optimal body performance.[19] Accordingly, DNA fragmentation percentage indicates both apoptosis of muscle cell and circulating mononuclear cells were affected due to increased oxidative stress.[20] It has been previously shown that apoptotic morphology is increased in dystrophic (mdx mice) muscle and in cultured muscle cells.[21] Neutrophils recruit at a muscle damage sites in order to facilitate muscle repair through removal of tissue debris as well as by activation of satellite cells[22], although somehow they contribute for more tissue damage by oxidative stress.[23,24]

In the present study a significant increase in Bax in blood mononuclear cells was

associated with a significant decrease in Bcl-2. It is a widely accepted view that BAX overexpression promotes cell death in response to apoptotic stimuli, whereas Bcl-2 inhibits it[25,26] BAX and Bcl-2 can form heterodimers, and overexpression of one antagonizes the effect of the other.[27] Bax genes expresses a Bax, a pro-apoptotic Bcl-2 family member, can heterodimerize with either Bcl-2 or Bcl-xL to nullify their anti-apoptotic properties.[28] Increased Bax mRNA expression has been observed in aging human lymphocytes.[29,30] Exercise loading caused an increased expression in Bax localization in muscular dystrophy animal model.[31] Meaning that oxidative stress due to exercise in DMD brings damage similar to that observed in aging, giving further proof to the present study.

It can be speculated in the present study that the significant increase in tissue Fas detected in plasma as well as circulating lymphocytes' FasL in DMD patients compared to controls contribute to increased apoptosis in muscle cells and consequently DNA fragmentation detected in blood. Increase in Bax and decreased Bcl2 in circulating mononuclear cells of DMD patients compared to controls reflects the increase of oxidative stress in these patients.[23,24] To our knowledge this is the first attempt to measure plasma Fas and Bax, Bcl2 and FasL in circulating lymphocytes of DMD patients. Results indicate that apoptosis and its markers determined in blood of DMD patients can replace the invasive technique of tissue biopsy.

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