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DUCHENNE MUSCULAR DYSTROPHY

FOURTH EDITION



ALAN E H EMERY, FRANCESCO MUNTONI
AND ROSALINE QUINLIVAN



Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy

FOURTH EDITION

Alan E H Emery
Francesco Muntoni
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Preface

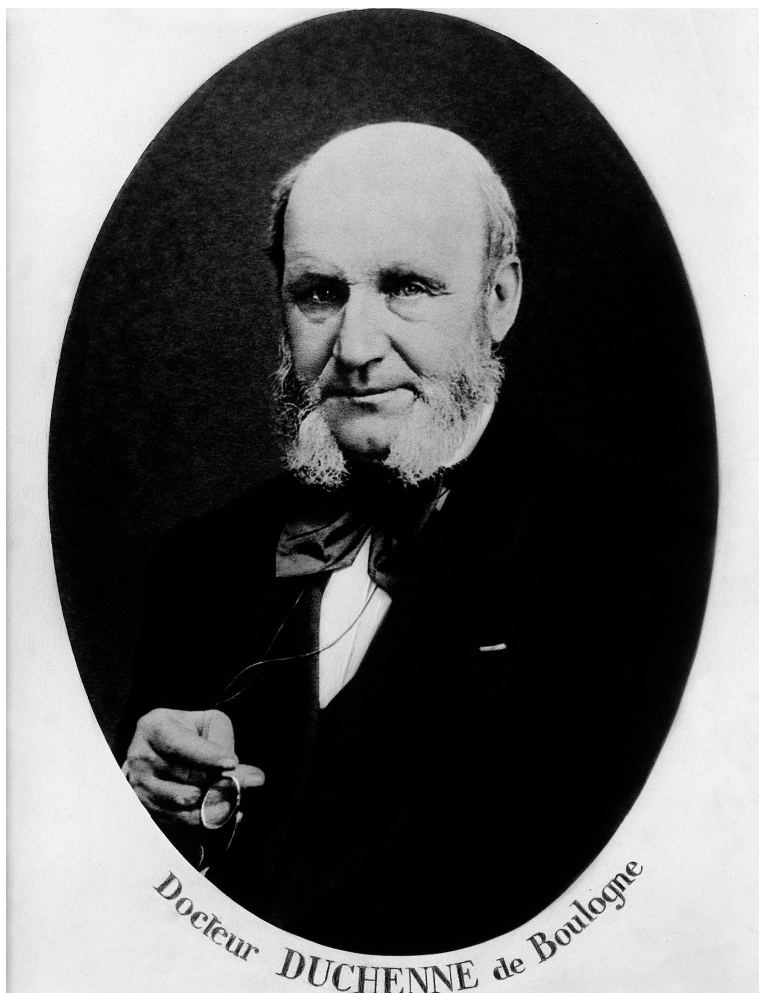
The first edition of this book was published in 1987, at the time the gene for Duchenne muscular dystrophy had been localized to the short arm of the X chromosome. However, shortly after publication, the gene itself was isolated and cloned, and its product dystrophin identified. This revolution in the history of the subject precipitated the production of a revised edition the following year! Over the subsequent 25 years, the subject has advanced considerably. There now seems a real possibility of some form of effective gene therapy in the not too distant future. Furthermore, these studies have subsequently been applied to other conditions, with the result that now some 50 different forms of dystrophy have been identified, and, in most of these disorders, the responsible gene and its protein product are now known.

In the preparation of this book, we should like to thank our various colleagues for information and help. In this regard, we are particularly grateful to Professor Caroline Sewry from Great Ormond Street Hospital for her help on specific aspects related to muscle pathology, to Dr Valeria Ricotti and Dr Adnan Manzur also from Great Ormond Street Hospital, to Dr Anna Mayhew from Newcastle University, and to all the colleagues of the UK North Star Network for their contribution to the longitudinal data collection on more than 600 DMD boys followed in the UK (http://www.muscular-dystrophy.org/how_we_help_you_for_professionals/clinical_databases). The support of the Muscular Dystrophy Campaign towards the North Star Network is also gratefully acknowledged, as the support of both the MRC Translational Research Centre and Great Ormond Street Hospital Biomedical Research Centre towards the activities of R. Q. and F. M.

We should also like to thank Caroline Smith of Oxford University Press for her professional and tireless help in the editing of this new edition.

We have endeavoured on occasions to draw attention to areas which may indicate possible paths for future research. However, as in previous editions, we have emphasized developments particularly relevant to the diagnosis, management, and treatment of Duchenne muscular dystrophy.

Oxford/Exeter, A. E. H. E.
London, F. M. and R. Q.



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Symbols and abbreviations

α	alpha	DAPC	dystrophin-associated glycoprotein complex
β	beta	der	derived
δ	delta	DEXA	dual-energy X-ray absorptiometry
γ	gamma	DMD	Duchenne muscular dystrophy
μ	mu	DNA	deoxyribonucleic acid
θ	theta	ECG	electrocardiogram
\sim	approximately	EEG	electroencephalography
$^{\circ}$	degree	EK	Egen Klassifikation
=	equal to	EMG	electromyography
\leq	equal to or less than	ENMC	European Neuromuscular Centre
<	less than	FIGE	field inversion gel electrophoresis
>	more than	FVC	forced vital capacity
\pm	plus or minus	g	gram
®	registered trademark	GABA	gamma-aminobutyric acid
AAV	adeno-associated virus	GOT	glutamic-oxaloacetic transaminase
ACE	angiotensin-converting enzyme	GPT	glutamic-pyruvic transaminase
ADHD	attention-deficit/hyperactivity disorder	HFDR	high-frequency deletion region
ADP	adenosine diphosphate	HFMD	hypertrophic feline muscular dystrophy
AMP	adenosine monophosphate	HLA	human leucocyte antigen
ATP	adenosine triphosphate	Ig	immunoglobulin
bFGF	basic fibroblast growth factor	IGF	insulin-like growth factor
BMD	Becker muscular dystrophy	IGF-1	insulin-like growth factor-1
bp	base pair	IGFBP	insulin growth factor-binding protein
Ca^{2+}	calcium ion	IQ	intelligence quotient
$[\text{Ca}^{2+}]_i$	free intracellular calcium	IU	international unit
CAMS	children and adolescent mental health services	IV	intravenous
cDNA	complementary deoxyribonucleic acid	K ⁺	potassium ion
CK	creatine kinase	KAFO	knee–ankle–foot orthosis
cM	centimorgan	kb	kilobase
CMD	congenital muscular dystrophy	kDa	kilodalton
CT	computerized tomography		
Da	dalton		

kg	kilogram	NSAA	North Star Ambulatory Assessment
L	litre	ORF	open reading frame
LAMA2	laminin alpha 2	OTC	ornithine transcarbamylase
LDH	lactate dehydrogenase	p	probability
LGMD	limb-girdle muscular dystrophy	$p\text{CO}_2$	carbon dioxide tension
MAPH	multiplex amplifiable probe hybridization	PCR	polymerase chain reaction
MAPK	mitogen-activated protein kinase	PERT	phenol-enhanced reassociation technique
mb	megabase	PET	positron emission tomography
MDC1A	muscular dystrophy congenital 1A	PFGE	pulsed-field gel electrophoresis
mg	milligram	PK	pyruvate kinase
Mg^{2+}	magnesium ion	$p\text{O}_2$	arterial oxygen tension
MHC	major histocompatibility complex	PTT	protein truncation test
MLPA	multiplex ligation-dependent probe amplification	r	correlation coefficient
mm	millimetre	RFLP	restriction fragment length polymorphism
MMP	matrix metalloproteinase	RNA	ribonucleic acid
MRC	Medical Research Council	SAPK-3	stress-activated protein kinase-3
MRI	magnetic resonance imaging	SCARMD	severe childhood autosomal recessive muscular dystrophy
mRNA	messenger ribonucleic acid	SCK	serum creatine kinase
MRS	magnetic resonance spectroscopy	SD	standard deviation
MW	molecular weight	SMA	spinal muscular atrophy
MZ	monozygotic	SNP	single nucleotide polymorphism
N	number	SPECT	single-photon emission computerized tomography
Na^+	sodium ion	SSCP	single-strand conformation polymorphism
NADP	nicotinamide adenine dinucleotide phosphate	TGF	transforming growth factor
NICE	National Institute for Health and Care Excellence	TRP	transient receptor potential
NIPPV	nasal intermittent positive pressure ventilation	U	unit
NIV	non-invasive ventilation	UK	United Kingdom
NMR	nuclear magnetic resonance	US	United States
nNOS	neuronal nitric oxide synthase	WFA	Wheelchair Football Association
NO	nitric oxide	Xce	X chromosome-controlling element
NOS	nitric oxide synthase	XLDCM	X-linked dilated cardiomyopathy

Chapter 1

Introduction to Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is the second most common single gene disorder in Western countries and has been recognized as a distinct entity for over 100 years. In the last 40 years or so, it has generated a great deal of interest among research workers, and, in their bibliography of what they considered to be the more important publications on the subject up to 1985, Herrmann and Spiegler (1985) listed no fewer than 789 references. Yet, despite all the interest, the cause remained elusive until 1987. This was, in part, due to the fact that the tissue that is predominantly affected, namely, the skeletal muscle, is complex, as must also be the genetic repertoire responsible for its normal development and functioning.

There are 434 different muscles in the human body, which, in the adult, contribute to over 40% of the total body weight. Much has been written on the development and morphology of muscle, but here only some general principles need be emphasized. The essential element of muscle is the muscle fibre (myofibre), which has been defined as ‘... a multinucleated cell that contains a large number of myofibrils embedded in a matrix of undifferentiated protoplasm, all enclosed within a fine sheath, the sarcolemma’. Muscle fibres are grouped together into fascicles, and a network of collagen fibres surrounds each fascicle (perimysium) and extends between individual muscle fibres (endomysium). Each of the muscle fibres, which vary in length from one muscle to another, is bounded by the plasma membrane (plasmalemma) and an outer basement membrane (basal lamina). The latter, along with the endomysium, constitutes the sarcolemma, though this term is sometimes also used when referring to the plasma and basement membranes together (see Fig. 1.1). Each multinucleated muscle fibre is formed during development by the fusion of several dividing mononucleated myoblasts derived from the myotomes. After fusion, the nuclei of the fibre do not divide again and lie in the cytoplasm (sarcoplasm), along with the contractile elements. Small mononucleated satellite cells are situated between the plasma and basement membranes of the muscle fibre. These cells are believed to be a persistent population of myoblastic stem cells that retain the ability to divide and are a source of additional muscle fibre nuclei during growth

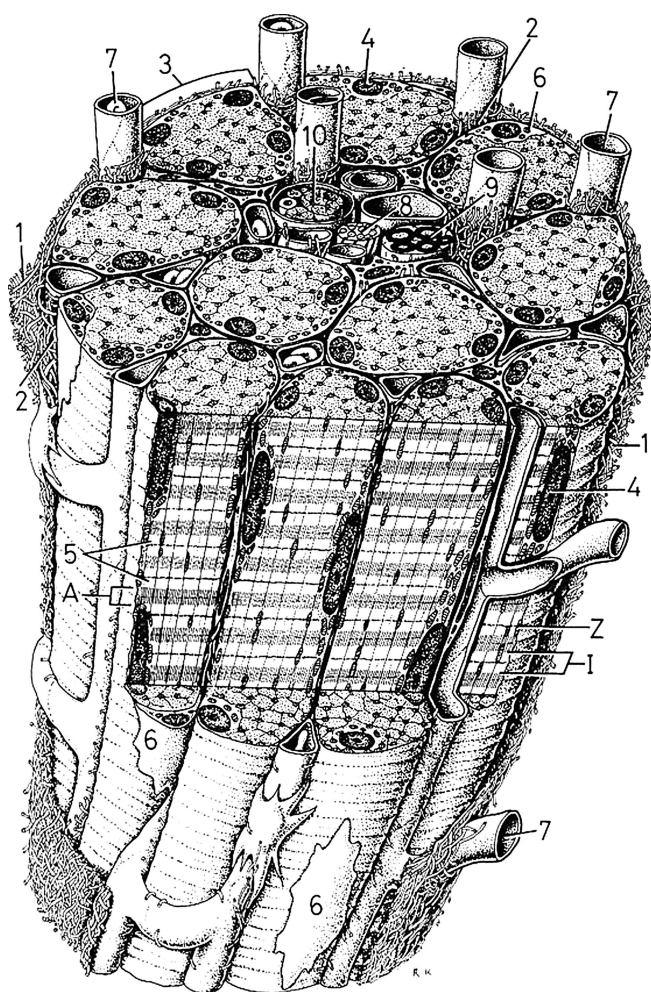


Fig. 1.1 Diagrammatic representation of a small fascicle of muscle fibres. 1, perimysium; 2, endomysium; 3, muscle fibre (myofibre); 4, nucleus; 5, contractile myofibrils; 6, satellite cells; 7, capillaries; A, dark bands; I, light bands; Z, Z-line.

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and regenerative repair (see Fig. 1.2). Recently, other progenitors located outside the basal lamina, including pericytes, endothelial cells, and interstitial cells, have been shown to have myogenic potential *in vitro* or after transplantation. The developmental origin of these progenitors is unclear, as is their lineage relationship with satellite cells, even though they may feed, to some extent, into the satellite cell compartment.

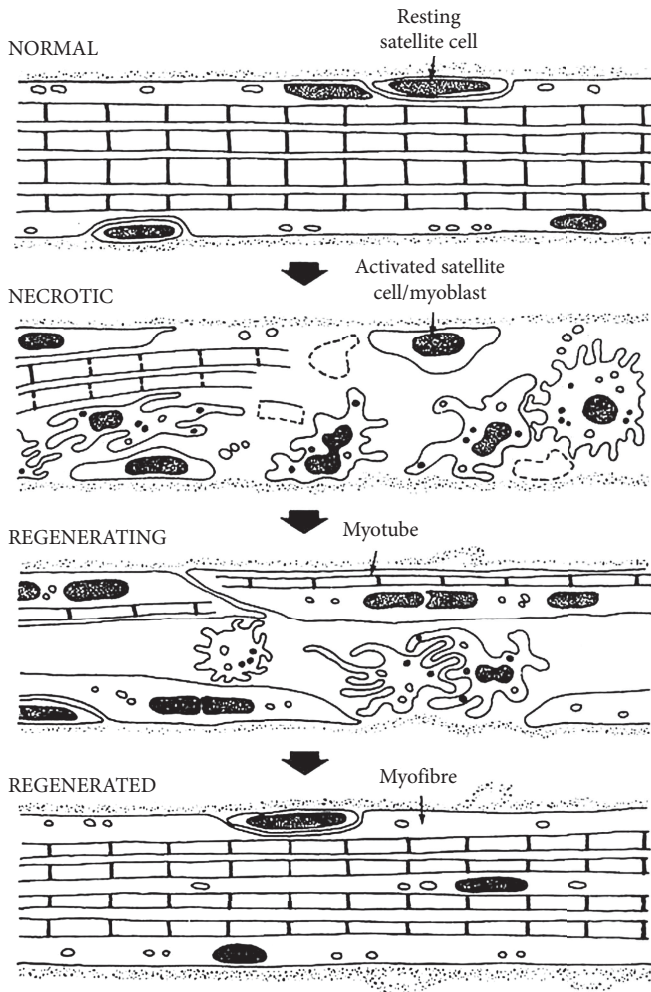


Fig. 1.2 Diagrammatic representation of a satellite cell and its role in muscle fibre regeneration.

Reproduced by kind permission of Professor M. J. Cullen.

A muscle fibre contains many myofibrils, which are the contractile elements of muscle and display alternating dark (A) and light (I) bands, and, through the centre of the latter, is the dense Z-line (band or disc) (see Fig. 1.1). The myofibrils are themselves composed of thick and thin myofilaments. During contraction and relaxation of the muscle, thin filaments slide between the thick filaments. In addition to nuclei and myofibrils, the sarcoplasm of a muscle fibre contains mitochondria, glycogen granules, lipid bodies, ribosomes, the transverse system of tubules (T-system), and the sarcoplasmic reticulum. The latter

is equivalent to the endoplasmic reticulum of cells in other tissues and forms a network of tubules that run between the myofibrils. The T-system consists of transversely arranged, fine, interconnecting, tubular extensions of the plasma membrane. A single T-tubule and two dilated ends of the sarcoplasmic reticulum form the so-called 'triads' . . . , which are concerned with the excitation and contraction of muscle fibres.

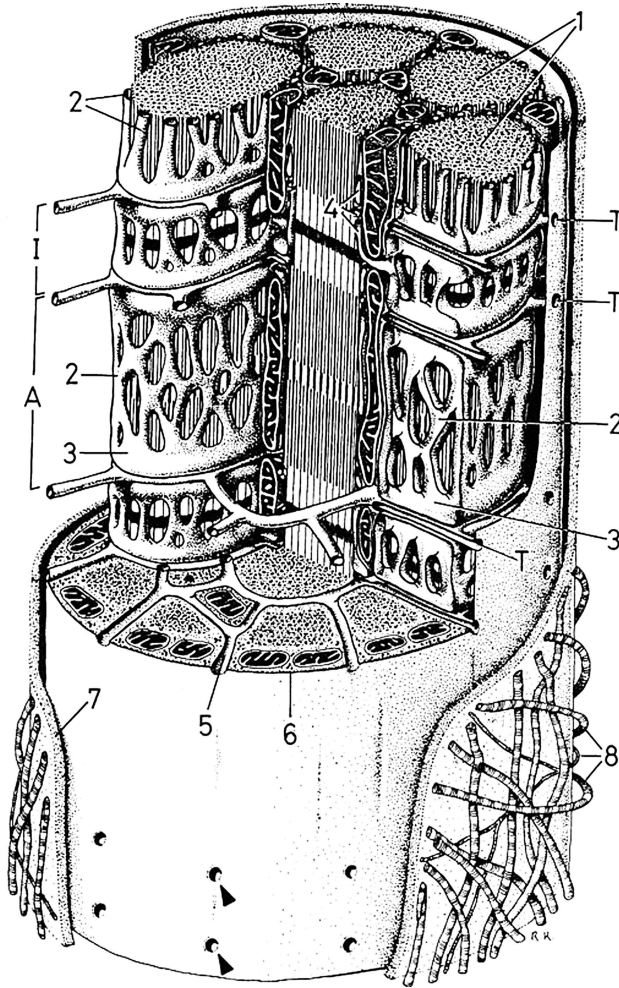


Fig. 1.3 Diagrammatic representation of a single muscle fibre. 1, myofibril; 2 and 3, sarcoplasmic reticulum; T and 5, transverse tubular system; 4, triads; 6, plasma membrane; 7, basement membrane; 8, endomysium.

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A nerve impulse, via the neuromuscular junction, produces depolarization of the muscle cell surface membrane, which then spreads inwards along the T-system to the triads. This results in the rapid release of calcium from the sarcoplasmic reticulum into the sarcoplasm, which, in turn, then results in the interaction between thick and thin filaments, producing muscle contraction (see Fig. 1.3).

Despite our increased understanding of the pathogenesis of DMD and Becker muscular dystrophy (BMD), we are still not certain about the extent to which different mechanisms lead to progressive muscle degeneration in these disorders. It is of interest to go through the mass of experimental information generated during the last decades on these dystrophies and compare it with our current knowledge about dystrophin and its localization and function. It is now clear that some of the data generated in the past are inconsistent with the current picture of this disease. However, some of the old data are intriguing, and, during this edition of the book, we have referred quite extensively to this previous work, wherever we felt it was of relevance.

During the process of selecting and emphasizing certain findings for the sake of clarity, it is inevitable that the resultant picture may be oversimplified, somewhat idiosyncratic, and probably inconsistent. The only defence is that voiced by Miguel de Unamuno in Erwin Schrödinger's (1944) book *What is life?*, which Watson and Crick and many others found so illuminating: 'If a man never contradicts himself, the reason must be that he virtually never says anything at all.'

A limited number of selected seminal references are quoted in each chapter, together with some of historical interest.

This book, which is partly based on cases and families studied by the authors, is not intended for the expert in any particular field, but rather for those with more catholic interests who are involved in this distressing and perplexing disease, which Gowers himself in 1879 referred to as being '... one of the most interesting and at the same time most sad.'

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Chapter 2

History of the disease

Early beginnings

The history of the disease is an interesting one and was traced in detail in Emery and Emery (2011). Muscular dystrophy has no doubt afflicted humans from the earliest times. Since the ancient Egyptians in their wall paintings often depicted physical abnormalities with some care, so that they can often be identified as diseases we now recognize, such as paralytic poliomyelitis and congenital dwarfism, it is just possible that they might have portrayed muscular dystrophy. In fact, it was suggested by the late Professor Becker that this might be so in a relief painting on the wall of a tomb in ancient Egypt, dating from the Eighteenth Dynasty of the New Kingdom, that is, circa 1500 BC (see Fig. 2.1). The subject depicted on the wall of the Temple of Hatshepsut is the Queen of Punt who shows lumbar lordosis and who, it has even been suggested, may also have some calf enlargement. However, in comparison with the adjoining figure, she seems generally fatter, and perhaps what is shown is no more than generalized obesity.

However, on the wall of a tomb at Beni Hasan (illustrated in manuscripts at the Ashmolean Museum, Oxford), dating from the Middle Kingdom (circa 2800–2500 BC), one of us noticed that there are depicted two figures of interest (see Fig. 2.2). The first has bilateral club foot. In the middle, however, is a boy with what could just possibly be muscular dystrophy. He has lost the normal arch of his feet, which is usually clear in Egyptian wall paintings, as seen in the figure to the right. Also, his calves seem somewhat enlarged, and he may have some degree of (pseudo)hypertrophy of certain upper limb muscles. On the other hand, as the hieroglyph above his head implies, he may have been a dwarf.

The *Transfiguration* was Raphael's last great work and was unfinished when he died on Good Friday in 1520, at the untimely age of 37. Vasari (1568), in his *Lives of the artists*, considers the boy in the painting to be 'possessed by a devil', an idea that may have prompted subsequent observers to suggest that it could illustrate a case of epilepsy. However, Duchenne himself, after whom the most common form of muscular dystrophy is named, when visiting National Hospital for Nervous Diseases in London, where a reproduction of the painting hung,

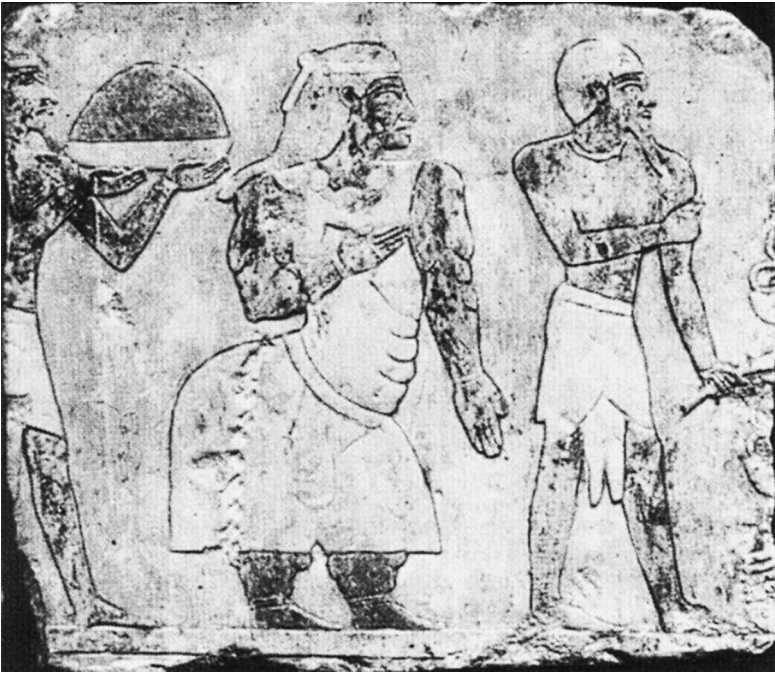


Fig. 2.1 Egyptian relief painting from the Eighteenth Dynasty.

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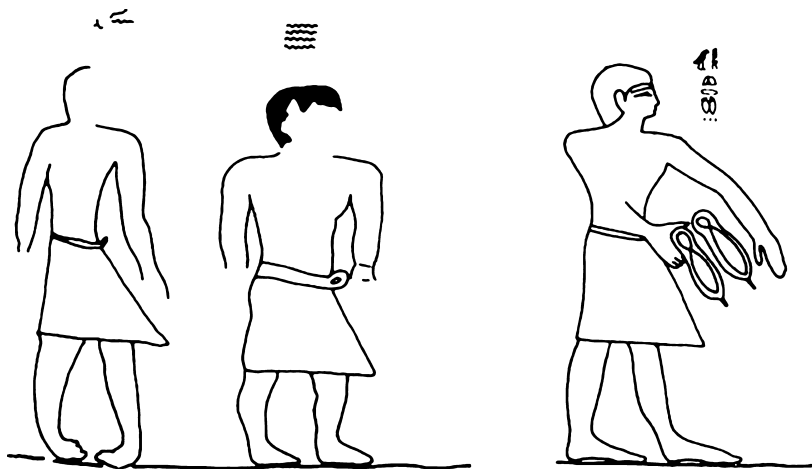


Fig. 2.2 Drawings from a tomb at Beni Hasan (circa 2800–2500 BC).

commented at the time that the boy depicted by the artist might be suffering from pseudohypertrophic muscular dystrophy.

It is also interesting to note that William Harvey (1578–1657) should be remembered not only for his observations on the circulation of the blood, but also for his studies in neurology and the structure and function of muscles. He showed, for example, that muscles can be distinguished by their structure, according to whether they are primarily fleshy, tendinous, sinewy, or membranous, as well as their action in causing movement.

However, the first clinical descriptions of dystrophy itself, at least in the English language, can be attributed to Charles Bell. He was born in Fountainbridge in Edinburgh in 1774 where he studied medicine and subsequently worked as a surgeon–anatomist, often illustrating his works with his own carefully executed drawings. At the age of 30 years, he moved to London, where he spent most of his working life, and was a founder of the Middlesex Medical School. He returned to Edinburgh to Chair of Surgery in 1835 and died in 1842 from angina. He is best remembered for being the first to describe paralysis of the facial nerve (Bell's palsy) and, with the French experimental physiologist François Magendie, for discovering the distinct functions of the posterior (sensory) and anterior (motor) nerve roots of the spinal cord.

Among his numerous publications is *The nervous system of the human body*. In it, he describes (Case 89) an 18-year-old man with wasting and weakness of the quadriceps muscles that had begun some 8 years previously and that:

... disabled him from rising; and it is now curious to observe how he will twist and jerk his body to throw himself upright from his seat. I use this expression, for it is a very different motion from that of rising from the chair. (Bell 1830, p. CLXIII)

There was no sensory loss. Without muscle pathology, the diagnosis cannot be certain, but the description would certainly be compatible with muscular dystrophy.

Gowers (1879), whose seminal contributions to the subject will be dealt with in more detail later, refers to the possibility of the disease having been described in 1838 by Coste and Gioja in the *Annali Clinici dell'Ospedale degli Incurabili di Napoli* which was abstracted in Schmidt's *Jahrbücher*. But this was a mistake. Recent research by Professor Giovanni Nigro of University of Naples (Nigro 1986) has revealed that the cases in question were presented by Professor Gaetano Conte (**not** Coste), with the help of a Dr L. Gioja, and reported in the journal, in fact, in 1836. Two brothers apparently first manifested the disease at age 8, had enlarged calves and progressive muscle wasting and weakness, which particularly affected the lower limbs, and subsequently developed contractures of the knees and hips. The elder brother died of cardiac failure. Sensory functions were intact, and mentation

was normal. The clinical features are presented in detail and the original publication has now been reproduced in full in *Cardiomyology*, Vol. V, no. 1, 1986, pp. 1–30.

It seems very likely that these two brothers probably had muscular dystrophy, though there is no report of muscle pathology. But certainly Professor Gaetano Conte, who was born in Naples in 1798 and dedicated most of his life to the study of ‘scrofole’ (? dystrophy), must rank among the pioneers in the history of the subject.

However, in 1847, a Mr Partridge presented a case to the Pathological Society of London (reported in the *London Medical Gazette* Vol. 5, p. 944) of a boy who, from about the age of 9 years, had developed progressive muscle wasting and weakness, had enlarged calves and muscle contractures, and died after an attack of measles at age 14. Examination of muscle tissue at autopsy revealed widespread fatty degeneration. In the same year, 1847, Dr W. J. Little, a physician at the London Hospital, studied two affected brothers aged 12 and 14, whom he reported in detail in 1853 in a book entitled *On the nature and treatment of the deformities of the human frame*. Both brothers presented a similar picture. Onset was in early childhood, with a tendency to walk on the toes and a peculiar gait with the ‘. . . head and body having been inclined backwards.’ There was progressive muscle wasting and weakness affecting the neck, trunk, and upper and lower extremities, associated with enlargement of the calf muscles and contractures ‘behind the heels.’ Sensation was normal. Both boys were unable to walk by the age of 11. The elder died at 14, and, at autopsy, examination of the gastrocnemius and soleus muscles (and some other muscles as well) revealed that the muscle tissue had been largely replaced by fat (‘adipose degeneration’). The brain and spinal cord appeared normal. These findings would certainly be consistent with the diagnosis of the severe form of muscular dystrophy that predominantly affects boys. However, the fullest and earliest description of this disorder must clearly be credited to Dr Edward Meryon of St Thomas’s Hospital, London (see Fig. 2.3).

Edward Meryon

Edward Meryon was born in 1809 and studied medicine in Paris and University College, London. He qualified as a Member of the Royal College of Surgeons in 1831, proceeding to an MD degree in 1844. His chief appointments were at St Thomas’s Hospital and Hospital for Nervous Diseases where it is just possible he may have been acquainted with the young William Gowers. He was apparently a man of wide learning and published several books relating to the nervous system. He also embarked on a *History of medicine* but unfortunately did not get beyond a first volume. In Feiling’s (1958) *History of the Maida Vale Hospital*, the

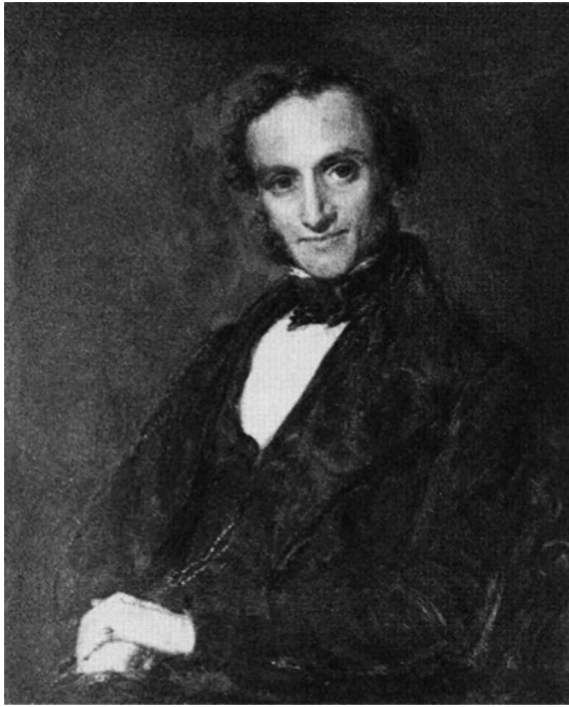


Fig. 2.3 Dr Edward Meryon.

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only reference to him reads: ‘Edward Meryon although not really distinguished in medicine, was clearly a well-known figure in London society at the time.’ He died at his home in Mayfair in 1880, at the age of 71 (Emery and Emery 2011).

In a communication addressed to the Royal Medical and Chirurgical Society in December 1851 and published in the *Transactions of the Society* the following year, Meryon described eight affected boys in three families. Interestingly, one of the two affected brothers in the second family is the case on which Partridge had earlier reported his autopsy findings in 1847. Meryon was particularly impressed by the familial nature of the condition and its predilection for males, and, in his book *Practical and pathological researches on the various forms of paralysis*, published in 1864, he details a family in which there were four affected cousins, with the disorder having been transmitted through three sisters. Second, he subjected muscle tissue to microscopic examination and reported that:

... the striped elementary primitive fibres were found to be completely destroyed, the sarcous element being diffused, and in many places converted into oil globules and

granular matter, whilst the sarcolemma and tunic of the elementary fibre was broken down and destroyed. (Meryon 1852, p. 76)

He therefore used the term ‘granular degeneration’ for the microscopic changes he observed. Furthermore, his emphasis on the subsarcolemma being broken down was certainly prescient. Some 130 years later, the protein defect was shown to reside in the sarcolemma! Third, he observed that:

... the relative proportion of the grey matter to the white in the cord, and the ganglionic cells of the former, and the tubular structure of the latter, as well as of the nerves and the white substance within the neurolemma, wherever examined by the microscope, all bore evidence of the healthy condition of the nervous system. (Meryon 1852, p. 78)

Thus, he concluded that this was a familial disease with a predilection for males, which primarily affected muscle tissue and was not a disease of the nervous system. Meryon’s clear delineation of the disorder and his understanding of its nature were very significant contributions. It is therefore unfortunate that he has not always been given the credit he deserves and that his work is completely overshadowed by that of Duchenne.

Duchenne de Boulogne

Guillaume Benjamin Amand Duchenne, Duchenne de Boulogne as he signed himself in order not to be confused with Duchesne of Paris, was born in the town of Boulogne-sur-Mer on 17 September 1806. He studied medicine in Paris where his teachers included Cruveilhier, Dupuytren, and Laennec. He then returned to Boulogne with the intention of being a family doctor. However, this proved a very unhappy time, for his young wife died of puerperal sepsis 14 days after giving birth to their son Émile in 1833, and, for several years afterwards, he remained depressed and lost interest in his work.

In 1839, he remarried, this time to a widow, but this does not seem to have been a happy marriage. Then, in 1842, at the age of 36, he returned to Paris where he spent the rest of his life. It has been suggested there may have been three factors instrumental in his return to Paris and to neurology: his growing interest in the possible therapeutic effects of electricity, his own family history of a ‘nervous’ disease, and his disastrous second marriage. Whatever the reasons, he quickly settled in Paris where he became a sort of itinerant physician, mainly at the Salpêtrière. He never held an official hospital or academic appointment and was therefore completely free to pursue his obsessional interests in the electrical stimulation of muscle, muscle function, and neuromuscular diseases. He studied the mechanisms of facial expression, a subject that had also interested Charles Bell some years previously. His painstaking observations led to clear descriptions of several disorders, his name now being most

closely associated with progressive muscular atrophy (with Aran) and progressive bulbar palsy (both part of the motor neuron disease complex), and, of course, pseudohypertrophic muscular dystrophy. He devised a strength gauge or dynamometer and a special needle-harpoon for muscle biopsy. The last 5 years of his life saw him famous but tragic: his wife died in 1870, and his son shortly afterwards from typhoid fever. He suffered a cerebral haemorrhage in August 1875, and Potain and Charcot never left him during the last weeks of his illness, taking it in turns to sleep by his bed. He died on 17 September 1875 on his 69th birthday. On 30 October, the Paris correspondent of *The Lancet*, commenting on Duchenne's life and work, wrote that, despite many adverse circumstances:

... his reputation has come out clear and bright as an honest, hard-working, acute, and ingenious observer, an original discoverer, a skilful professional man, and a kind-hearted, benevolent gentleman.

Despite his abounding interest in research, it seems he never lost a bedside manner.

Duchenne's interest in muscular dystrophy was first aroused in 1858 when his attention was drawn to a case, details of which he published in 1861 in the second edition of his book *De l'électrisation localisée* (Duchenne 1861). Later, in 1868, he reviewed in considerable detail his original case plus 12 further cases, two of whom were young girls, and referred to a further 15 cases in the German literature (Duchenne 1868). By 1870, he had seen some 40 cases of the disease, not counting those he saw when he visited the London hospitals around this time (see Fig. 2.4).

Duchenne defined the disease as being characterized by: progressive weakness of movement, first affecting the lower limbs and then later the upper limbs; a gradual increase in the size of many affected muscles; an increase in interstitial connective tissue in affected muscles, with the production of abundant fibrous and adipose tissue in the later stages.

Though Meryon had studied the histology of affected muscles, his observations had been limited to material obtained at autopsy. Duchenne, on the other hand, used his needle-harpoon (*emporte-pièce histologique*) to obtain biopsy specimens in life. In fact, using this technique, he was able to study material from the same patient at different stages of the disease. His observations led him to conclude that the fundamental anatomical lesion was hyperplasia of the interstitial connective tissue which therefore prompted him to use the term *paralysie myosclérosique* as an alternative to *paralysie musculaire pseudohypertrophique*. Previously, a pathological diagnosis could only be made at autopsy, the so-called diagnosis of Morgagni. But Duchenne's technique meant that

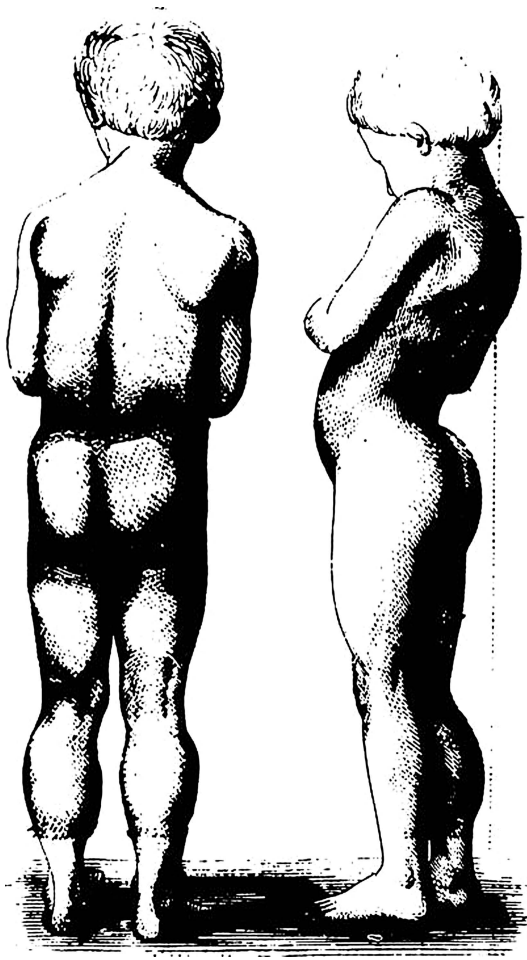


Fig. 2.4 Duchenne's original case, showing marked calf enlargement and lumbar lordosis.

Reproduced from Duchenne, G. B. A., *Recherches sur la paralysie musculaire pseudohypertrophique ou paralysie myo-sclérosique*. *Archives Générales de Médecine*, Volume 11, p. 8, 1868.

such a diagnosis could be made in life. He believed correctly that, unlike progressive (spinal) muscular atrophy of childhood, the disease was not caused by a lesion in the spinal cord. In this matter, it is rather disappointing that Duchenne felt he should dismiss Meryon's contributions when he says that the latter confused the disease with progressive muscular atrophy, and therefore thought it had a neurogenic basis, which, as we have seen, he did not, and Duchenne goes further by giving the date of Meryon's address to the Royal Medical and Chirurgical Society as 1866 when, in fact, it was some 15 years earlier in 1851. Duchenne carefully weighed the available evidence regarding the possible aetiology of the disorder, particularly with regard to possible neurological or vasomotor factors, but had to conclude, just as we would have done until

relatively recently, ‘... *la pathogénie de la paralysie pseudo-hypertrophique est très obscure; elle doit être réservée...*’

Though, as we have seen, Meryon had made a special note of his observation that the sarcolemma was broken down, a singularly important point, we now know that the primary defect does, in fact, reside in the sarcolemma.

William R. Gowers

Considerable interest now began to be shown in the disease, and numerous case reports appeared in the French, English, German, American, Australian, and Danish literature. However, the next physician to enter the stage who made a significant contribution to the subject was William R. Gowers. Gowers was born in 1845 and spent all his life in London. He had a brilliant undergraduate career at University College Hospital where he was awarded medals in almost every subject of the medical curriculum and graduated with first class honours. He later became Professor of Medicine at University College, as well as being a physician at National Hospital for Nervous Diseases. He was a man of immense intellect and wide interests. He was a knowledgeable botanist and an authority on mosses, an accomplished artist (he exhibited at Royal Academy), and an obsessional shorthand writer. He introduced into medicine a number of new terms such as ‘knee-jerk’, ‘fibrositis’, and ‘abiotrophy’. He described several clinical signs, including the nasal smile in myasthenia gravis, as well as the so-called Gowers’ manoeuvre. He also invented a haemocytometer that was widely used for many years. It is understandable that, in his day, he was therefore widely admired and respected. He remained, however, a reserved and very private individual with few intimate friends. He died in 1915 at the age of 70 (see Fig. 2.5).

Gowers’s interest in muscular dystrophy was kindled when working as a premedical student apprentice to Dr Thomas Simpson in Coggeshall, Essex. Here, he came across a family with four brothers afflicted with a ‘strange disorder of locomotion with wasting of some muscles and enlargement of others’. Later, he learned that the disease had been described in 1852 by Meryon, and, in 1879, he delivered a series of lectures on the disorder at National Hospital which were published in *The Lancet* and subsequently made into a monograph (Gowers 1879). The latter was based on information from 220 cases, which included 24 he had seen himself, 20 seen by colleagues, and the remainder from the literature. In deference to Duchenne, he referred to the disease as ‘pseudohypertrophic muscular paralysis’, and, in his monograph, he attempted to give as complete a picture of the disease as possible, with detailed discussions of the clinical features, pathology, prognosis, and

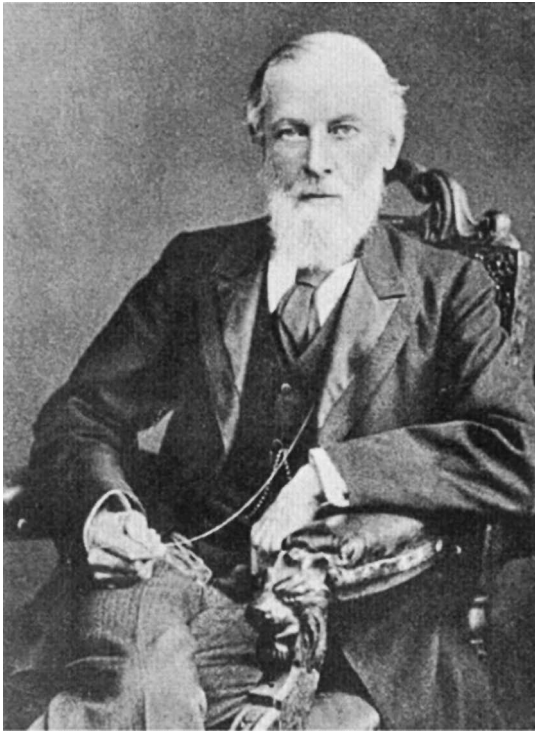


Fig. 2.5 Sir William Gowers.

Reproduced by kind permission of Dr Macdonald Critchley.

possible treatment. As with all of Gowers's writings, clarity, thoughtfulness, and good prose are evident. This is illustrated in the graphic opening paragraph (Gowers 1879):

The disease is one of the most interesting and at the same time most sad, of all those with which we have to deal: interesting on account of its peculiar features and mysterious nature; sad on account of our powerlessness to influence its course, except in a very slight degree, and on account of the conditions in which it occurs. It is a disease of early life and of early growth. Manifesting itself commonly at the transition from infancy to childhood, it develops with the child's development, grows with his growth—so that every increase in stature means an increase in weakness, and each year takes him a step further on the road to a helpless infirmity, and in most cases to an early and inevitable death.

The interest in the book lies mainly in the detailed presentation of the clinical features of the disease and describes what is nowadays usually referred to as the Gowers' manoeuvre or Gowers' sign (see Fig. 2.6). Weakness of the hip and

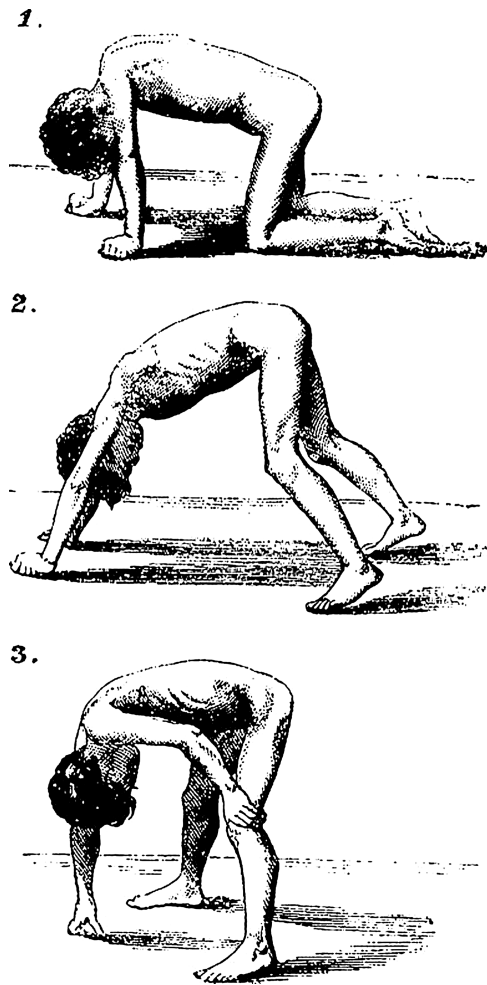


Fig. 2.6 Gowers' sign or manoeuvre.

Reproduced from Gowers, W. R., *Pseudo-hypertrophic muscular paralysis—a clinical lecture*, J. and A. Churchill, London, UK, 1879.

knee extensors causes difficulty in rising from the floor or a chair. As a result, when getting up, patients:

... first put the hands on the ground (1), then stretch out the legs behind them far apart, and, the chief weight of the trunk resting on the hands, by keeping the toes on the ground and pushing the body backwards, they manage to get the knees extended so that the trunk is supported by the hands and feet, all placed as widely apart as possible (2). Next the hands are moved alternately along the ground backwards so as to bring a larger portion of the weight of the trunk over the legs. Then one hand is placed upon the knee (3), and a push with this and with the other hand on the ground is sufficient to enable the extensors of the hip to bring the trunk into the upright posture.

Gowers recognized that this had also been noted by Duchenne: ‘If he bent forward he could only recover his position by catching hold of the furniture, or by supporting his hands on his thighs.’ At first, Gowers thought the action of putting the hands on the knees, then grasping the thighs higher and higher (‘climbing up his thighs’) so as to extend the hips and push up the trunk was pathognomonic for the disease. However, he later realized that it could also be seen in other diseases in which the same muscle groups were affected.

Gowers also emphasized that the disease was primarily a disease of muscle and that the spinal cord was unaffected. Further, he was impressed by the predilection for males and was clearly convinced of the hereditary nature of the disorder. Of the total of 220 cases, only 30 were females, and these were usually less severely affected. Although isolated cases were common, he was impressed by the frequency with which other relatives could be affected (of the 220 cases, 102 were isolated and 118 were grouped in 39 families). Perhaps his most revealing observation was that ‘. . . the disease is almost never to be heard of on the side of the father; when antecedent cases have occurred they have almost invariably been on the side of the mother.’ Gowers also observed that a woman could have affected sons by different husbands but found no instance in which members of the father’s family suffered from the disease. He concluded that limitation to males and inheritance only through the mother were the same as in haemophilia. This pattern of inheritance was already recognized at the time, although, in fact, it had been appreciated since the days of the *Talmud* some 1500 years earlier. The Jews excused from circumcision the sons of all the sisters of a mother who had a son with the ‘bleeding disease’. The sons of the father’s sibs were not so excused. The genetic basis for this mode of inheritance was appreciated through the rediscovery of Mendelism in 1900 and its cytological basis (X-linkage) recognized a few years later.

Wilhelm Heinrich Erb

By this stage, in the story, it was now quite clear that the disease primarily affected skeletal muscle and was hereditary. However, it was also clear that not all cases presented with exactly the same clinical features—females were occasionally affected, and sometimes the disease in males would pursue a more benign course, with survival into at least the third decade (for example, Gowers’s cases 23, 35, and 36). This raised the possibility that perhaps, after all, there was more than one disease, an idea first pursued by Erb.

Wilhelm Heinrich Erb was born in 1840 in Bavaria and studied medicine at Heidelberg, Erlangen, and Munich. His subsequent professional life was spent almost entirely in Heidelberg. Erb was, without doubt, one of the greatest

clinical neurologists of all time (Kuhn and Rüdel 1990). But he was also a great clinical teacher—the archetype of the time: severe, cultured, and always impeccably dressed. He died of a heart attack when he was 81—it is said whilst listening to Beethoven's *Eroica*.

Erb was greatly influenced by the studies of Duchenne, both with regard to the possible diagnostic and therapeutic uses of electricity in neurology, as well as his work on muscle disease. His pathological studies convinced him that the disease was due to a degeneration of muscle tissue, and he coined the term 'Dystrophia muscularis progressiva' or progressive muscular dystrophy, a term that has been used ever since (Erb 1884). Many of the cases he studied were clearly different from cases described by Duchenne, and he was well aware of this. In fact, he is credited with being the first to attempt to classify this group of diseases (Erb 1891). The details of his classification would now be questioned, but the idea that this was not one disease, but a heterogeneous group of disorders, was certainly true.

Recognition of heterogeneity

Over the next few decades, as physicians began to study their patients in increasing detail, attempts began to be made to categorize different types and to classify them according to various clinical criteria such as distribution of muscle weakness and age at onset and progression. Later, the mode of inheritance was added. Although there were a few who continued for a while to believe that muscular dystrophy was essentially one disease, this view was gradually abandoned. However, there is a serious problem in considering heterogeneity within a group of disorders such as the muscular dystrophies. Differences between disease entities may be more apparent than real—variations within a spectrum and not necessarily a reflection of true genetic differences. This is constantly to be borne in mind when attempting to resolve apparent heterogeneity. The sentiments of Francis Bacon in 1620 are therefore apt:

The steady and acute mind can fix its contemplations and dwell and fasten on the subtlest distinctions: the lofty and discursive mind recognises and puts together the finest and most general resemblances. Both kinds however easily err in excess, by catching the one at gradations the other at shadows.

How heterogeneity within this group of diseases was gradually resolved makes a fascinating byway in the history of medicine. However, there would be little value here in summarizing the detailed findings of these earlier studies, which, in any event, have been critically reviewed elsewhere (Emery and Emery 2011; Mercuri and Muntoni 2013). A classification, based on current information favoured by the authors, is reproduced in Table 2.1.

Table 2.1 Clinical, biochemical, and genetic classification of the muscular dystrophies due to known gene defects

Disease	Inheritance	OMIM	Locus	Gene symbol	Protein	Main localization
DMD/BMD	X-R	310 200/ 300 376	Xq21.2	<i>DMD</i>	Dystrophin	Sarcolemmal-associated protein
Limb-girdle muscular dystrophy (LGMD) type 1A	AD	159 000	5q31	<i>MYOT</i>	Myotilin	Sarcomeric-associated protein (Z-disc)
LGMD type 1B	AD	159 001	1q21.2	<i>LMNA</i>	Lamin A/C	Nuclear lamina-associated protein
LGMD type 1C	AD	607 780	3p25	<i>CAV3</i>	Caveolin-3	Sarcolemmal-associated protein
LGMD type 1D	AD	603 511	7q	<i>DNAJB6</i>	Co-chaperone DNAJB6	Sarcomeric-associated protein (Z-disc)
LGMD type 1E	AD	602 067	6q23	<i>DES</i>	Desmin	Intermediate filament protein
LGMD type 1F	AD	608 423	7q32	?	?	
LGMD type 1G	AD	609 115	4p21	?	?	
LGMD type 1H	AD	613 530	3p23-p25	?	?	
LGMD type 2A	AR	253 600	15q15.1	<i>CAPN3</i>	Calpain-3	Myofibril-associated proteins
LGMD type 2B	AR	253 601	2p13	<i>DYSF</i>	Dysferlin	Sarcolemmal-associated protein
LGMD type 2C	AR	253 700	13q12	<i>SGCG</i>	Y-sarcoglycan	Sarcolemmal-associated protein
LGMD type 2D	AR	608 099	17q12-q21.33	<i>SGCA</i>	α -sarcoglycan	Sarcolemmal-associated protein
LGMD type 2E	AR	604 286	4q12	<i>SGCB</i>	α -sarcoglycan	Sarcolemmal-associated protein
LGMD type 2F	AR	601 287	5q33	<i>SGCD</i>	α -sarcoglycan	Sarcolemmal-associated protein

Table 2.1 (continued) Clinical, biochemical, and genetic classification of the muscular dystrophies due to known gene defects

Disease	Inheritance	OMIM	Locus	Gene symbol	Protein	Main localization
LGMD type 2G	AR	601 954	17q12	<i>TCAP</i>	Titin-cap (telethonin)	Sarcomeric-associated protein (Z-disc)
LGMD type 2H	AR	254 110	9q31-q34	<i>TRIM32</i>	Tripartite motif-containing 32 (ubiquitin ligase)	Sarcomeric-associated protein (Z-disc)
LGMD type 2I	AR	607 155	19q13.3	<i>FKRP</i>	Fukutin-related protein	Putative glycosyltransferase enzymes
LGMD type 2J	AR	608 807	2q31	<i>TTN</i>	Titin	Sarcomeric protein
LGMD type 2K	AR	609 308	9q34	<i>POMT1</i>	Protein-1-O-mannosyl-transferase 1	Glycosyltransferase enzymes
LGMD type 2L	AR	611 307	11p14.3	<i>ANO5</i>	Anoctamin 5	Transmembrane protein, possible sarcoplasmic reticulum
LGMD type 2M	AR	611 588	9q31	<i>FKTN</i>	Fukutin	Putative glycosyltransferase enzymes
LGMD type 2N	AR	613 158	14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes
LGMD type 2O	AR	613 157	1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta 1,2-N-aminytransferase 1	Glycosyltransferase enzymes
LGMD type 2Q	AR	613 723	8q24	<i>PLEC1</i>	Plectin 1	Sarcomeric-associated protein (Z-disc)
LGMD type 2?	AR	613 818	3p21	<i>DAG1</i>	Dystrophin-associated glycoprotein 1	Sarcolemmal-associated protein
Facioscapulohumeral MD type 1	AD	158 900	4q35	?	DUX4 and chromatin rearrangement	

Facioscapulohumeral MD type 2	AD		18	?	SMCHD1	Structural maintenance of chromosomes flexible hinge domain-containing 1
Emery–Dreifuss MD X-linked type 1	X-R	310 300	Xq28	<i>EMD</i>	Emerin	Nuclear membrane protein
Emery–Dreifuss MD X-linked type 2	X-R	300 696	Xq27.2	<i>FHL1</i>	Four-and-a-half LIM domain 1	
Emery–Dreifuss MD autosomal dominant	AD	2 181 350	1q21.2	<i>LMNA</i>	Lamin A/C	Nuclear membrane protein
Emery Dreifuss MD autosomal recessive	AR	604 929	1q21.2	<i>LMNA</i>	Lamin A/C	Nuclear membrane protein
Emery–Dreifuss MD with Nesprin-1 defect	AD	612 998	6q25	<i>SYNE1</i>	Spectrin repeat containing, nuclear envelope 1 (Nesprin-1)	Nuclear membrane protein
Emery–Dreifuss MD with Nesprin-2 defect	AD	5 612 999	4q23	<i>SYNE2</i>	Spectrin repeat containing, nuclear envelope 2 (Nesprin-2)	Nuclear membrane protein
CMD with merosin deficiency (MDC1A)	AR	607 855	6q2	<i>LAMA2</i>	Laminin alpha 2 chain of merosin	Extracellular matrix proteins
CMD	AR	604 801	1q42	?	?	
CMD and abnormal glycosylation of dystroglycan (MDC1C)	AR	606 612	19q13	<i>FKRP</i>	Fukutin-related protein	Putative glycosyltransferase enzymes

Table 2.1 (continued) Clinical, biochemical, and genetic classification of the muscular dystrophies due to known gene defects

Disease	Inheritance	OMIM	Locus	Gene symbol	Protein	Main localization
CMD and abnormal glycosylation of dystroglycan (MDC1D)	AR	608 840	22q12	<i>LARGE</i>	Like-glycosyl transferase	Putative glycosyltransferase enzymes
Fukuyama CMD	AR	253 800	9q31-q33	<i>FCMD</i>	Fukutin	Putative glycosyltransferase enzymes
Walker Warburg syndrome	AR	236 670	9q31-q33	<i>FCMD</i>	Fukutin	Putative glycosyltransferase enzymes
		236 670	9q34	<i>POMT1</i>	Protein-1-O-mannosyl-transferase 1	Glycosyltransferase enzymes
		236 670	14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes
		236 670	1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta 1,2-N-aminytransferase 1	Glycosyltransferase enzymes
		236 670	19q13	<i>FKRP</i>	Fukutin-related protein	Putative glycosyltransferase enzymes
Muscle–eye–brain disease	AR	253 280	1p34	<i>POMGNT1</i>	Protein-O-linked mannose beta 1,2-N-aminytransferase 1	Glycosyltransferase enzymes
		253 280	19q13	<i>FKRP</i>	Fukutin-related protein	Putative glycosyltransferase enzymes
		253 280	14q24	<i>POMT2</i>	Protein-O-mannosyl-transferase 2	Glycosyltransferase enzymes

CMD due to glycosylation disorder	AR		9q34.1	<i>DPM2</i>	Dolichyl-phosphate mannosyltransferase polypeptide 2	Glycosyltransferase enzymes
			1q21.3	<i>DPM3</i>	Dolichyl-phosphate mannosyltransferase polypeptide 3	Glycosyltransferase enzymes
CMD with mitochondrial structural abnormalities	mtDNA	602 541	22q13	<i>CHKB</i>	Choline kinase	Sarcolemmal and mitochondrial membrane
CMD with rigid spine syndrome	AR	602 771	1p36	<i>SEPN1</i>	Selenoprotein N1	Endoplasmic reticulum protein
Ullrich syndrome	AR	254 090	21q22.3	<i>COL6A1</i>	Collagen type VI, subunit alpha 1	Extracellular matrix proteins
		254 090	21q22.3	<i>COL6A2</i>	Collagen type VI, subunit alpha 2	Extracellular matrix proteins
		254 090	2q37	<i>COL6A3</i>	Collagen type VI, subunit alpha 3	Extracellular matrix proteins
CMD with integrin $\alpha 7$ defect	AR	613 204	12q13	<i>ITGA7</i>	Integrin $\alpha 7$	External sarcolemmal protein
CMD with integrin $\alpha 9$ defect	AR		3p21.3	<i>ITGA9</i>	Integrin $\alpha 9$	External sarcolemmal protein

AD, autosomal dominant; AR, autosomal recessive; MD, muscular dystrophy; mtDNA, mitochondrial deoxyribonucleic acid; X-R, X-linked recessive.

At this point, perhaps it would be appropriate to consider which disorders are included under the heading ‘muscular dystrophies.’ For practical purposes, a useful definition is ‘a group of inherited disorders that are characterized by a progressive muscle wasting and weakness, in which the muscle histology has certain distinctive features (muscle fibre necrosis, phagocytosis, regeneration, fibrosis, etc.) and where there is no clinical or laboratory evidence of spinal cord or peripheral nervous system involvement or myotonia.’ Excluded therefore are the myotonic syndromes and the various congenital myopathies. However, such a definition encompasses disorders that vary considerably in their onset, severity, and distribution of muscle involvement. At one extreme, there is the rapidly progressive form of congenital muscular dystrophy (CMD), which is present at birth with generalized muscle involvement. At the other extreme, there is ocular muscular dystrophy where the onset is in adult life and the disease is often limited to the extra-ocular muscles and may be no more than a minor inconvenience.

In this book, we shall concentrate on that form of dystrophy associated with the name of Duchenne. Until fairly recently, eponyms were retained for several other related forms of dystrophy such as the scapulohumeral (Erb), pelvifemoral (Leyden—Möbius), and facioscapulohumeral (Landouzy—Dejerine) forms. But this habit has now been largely abandoned in favour of a clinical–genetic nomenclature. However, there remains one important exception—the retention of Becker’s name for the X-linked form of the disease that clinically resembles DMD but is more benign, with affected individuals often surviving into middle age.

Becker was, until his retirement in 1975, Professor of Human Genetics at University of Göttingen, a position he had held since 1957. He died in 2001 (see Fig. 2.7). Although, by training, a neurologist and psychiatrist, most of

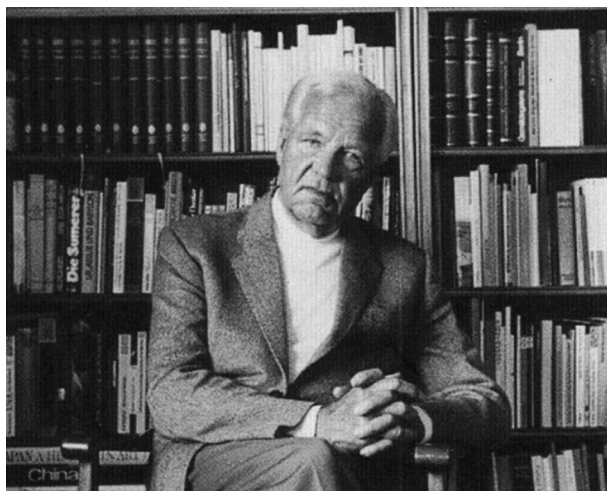


Fig. 2.7 The late Professor Peter Emil Becker.

Table 2.2 Landmarks in the history of DMD

Nineteenth century	DMD recognized as a specific clinical disorder (Conte and Gioja 1836; Meryon 1852; Duchenne 1861, 1868; Gowers 1879)
1955	BMD recognized as a distinct X-linked muscular dystrophy
1959–60	Serum creatine kinase (SCK) raised in patients and in female carriers
1978–83	DMD mapped to Xp21 by X/A translocations
1983–4	BMD and DMD shown to be allelic
1985	Gene-specific probes
1987–8	Gene deletions detected; complementary deoxyribonucleic acid (cDNA) cloned and sequenced Protein product (dystrophin) identified
1989–90	Dystrophin localization and functional studies begin Myoblast transfer experiments in mouse and humans First randomized controlled trials of glucocorticoids
1990–2	Gene transfer experiments in animal model
1992–4	Negative results of controlled studies on myoblast transfer in humans Identification of the dystrophin-like molecule utrophin Identification of the dystrophin-associated glycoprotein complex (DAPC) Proof of principle that antisense oligonucleotides can induce exon skipping and allow production of dystrophin in dystrophic cells, report on systematic use of non-invasive nocturnal ventilatory support on survival of DMD
1999	Possibility of stem cell therapy in animal models
2000–5	Systemic administration of antisense oligonucleotides in dystrophic animals Pharmacological read-through of non-sense mutations in DMD Phase I study of intramuscular plasmid delivery in DMD
2007	First report of use of antisense oligonucleotides to skip exon 51 administered intramuscularly in DMD boys
2008	Randomized, placebo-controlled study of a systemic drug to induce read-through non-sense mutations in DMD boys
2010	First phase I study using intramuscular administration of adenoviral vector
2011	Two reports of dystrophin restoration after systemic administration of antisense oligonucleotides to skip exon 51 in DMD boys Phase I clinical trial using systemically delivered autologous mesoangioblast
2013	Results of randomized, placebo-controlled studies on systemic administration of antisense oligonucleotides to skip exon 51 Initiation of phase I studies to skip exons 44, 45, and 53

his work was centred on human genetics. The dystrophy that bears his name was first brought to his attention by Dr Franz Kiener, a psychologist in Regensburg, who sought Becker's advice on the disease that had affected several of his own relatives. Together, they studied the family in detail (Becker and Kiener 1955), and, a few years later, Becker reported two further families with the same disease (Becker 1962). Patients with the disease had been observed previously by others, but it was Becker who showed that it was clearly a separate clinical entity. It is now known that Duchenne and Becker

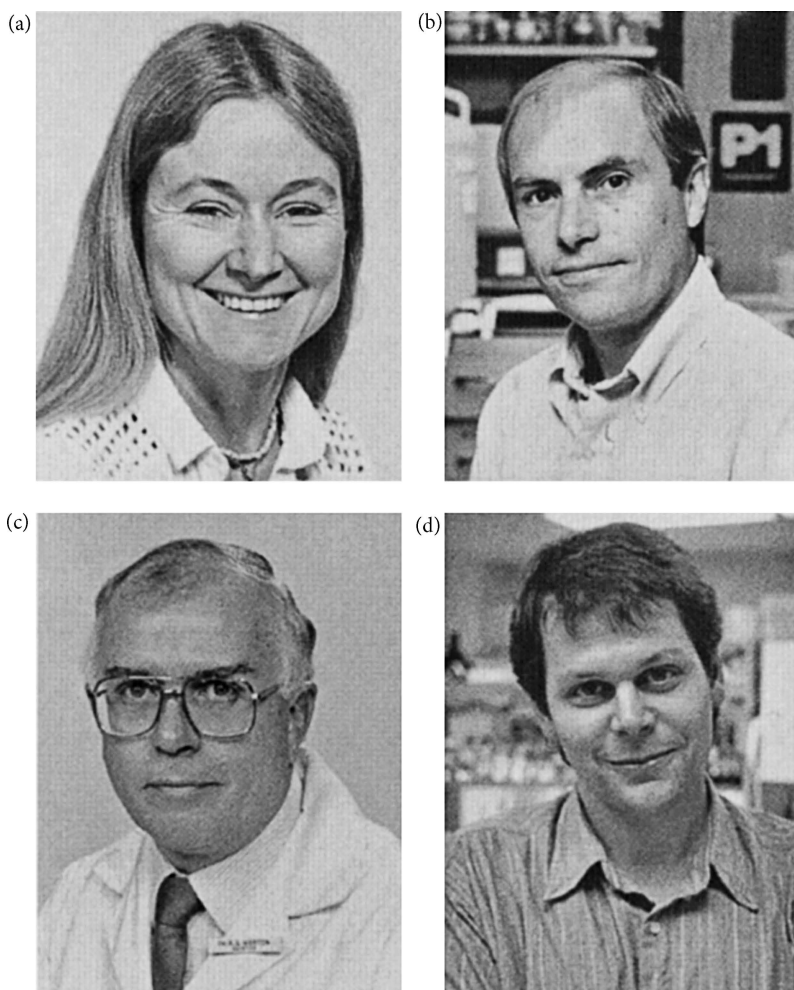


Fig. 2.8 Investigators who have played leading roles in recent research that has led to the localization and characterization of the Duchenne gene and its product. (a) Dr Kay Davies, (b) Dr Lou Kunkel, (c) Dr Ron Worton, and (d) Dr Eric Hoffman.

types of muscular dystrophy are due to mutations at the same locus on the X chromosome, that is, they are allelic.

Recent developments

Some important landmarks in the history of DMD are listed in Table 2.2. The recent history of DMD started in the late 1970s with the mapping of the defective gene to a specific locus on the short arm of the X chromosome (Xp21). Within a few years, gene-specific probes became available, culminating in the identification and characterization of the defective protein in DMD and BMD, namely, dystrophin. Many individuals have played important roles in these studies, the most notable being Dr Kay Davies of Oxford, Dr Lou Kunkel of Boston, Dr Ron Worton of Toronto, and Dr Eric Hoffman of Pittsburgh (see Fig. 2.8). These developments will be discussed in detail in the text.

Based on these various findings, rational new approaches to therapy are beginning to be considered. The prospects are now more hopeful than ever that, in the not too distant future, an effective therapy will be found for this tragic disease.

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Clinical features

Introduction to clinical features

The skeletal muscle of children with DMD functions quite well in the preclinical stages, despite the presence of severe histological changes in the muscle biopsy. It is, however, more fragile, compared to normal muscle, and cannot sustain its physiological function without being damaged. The weakness only starts to appear when a significant part of skeletal muscle has degenerated and been replaced by fibro-adipose tissue. As a result of this, the onset of clinical signs and symptoms related to weakness is insidious, and parents may be unaware that anything is wrong for some time, and physicians may find it difficult to appreciate the early features of the condition.

Onset

Occasionally, mothers volunteer that their affected son seemed ‘floppy’ at birth and in infancy. However, this is never as pronounced, or as frequent, as in the congenital forms of muscular dystrophy or infantile spinal muscular atrophy (SMA; Werdnig–Hoffmann disease).

In a careful follow-up study, some years ago, of 109 infants presenting with hypotonia at birth or shortly after birth, 60% were found to be affected by the severe form of SMA (Werdnig–Hoffmann disease), 20% by one of the congenital myopathies or CMDs, and the remainder by a variety of conditions, including cerebral palsy and mental handicap. Three of the 109 cases in the study went on to develop DMD.

The most common presentation is delay in walking and clumsy gait, with a tendency to walk on tiptoes. Of the 114 cases in which this information was reliably documented, in 64 (56%), walking was delayed until at least 18 months (see Table 3.1) and roughly a quarter did not walk until they were at least 2 years old. In normal children, by comparison, the average age for learning to walk is about 13 months, and 97% are walking by 18 months.

Approximate percentiles for age at apparent onset in 144 cases are given in Table 3.2. In this, and other age-related events based on cases studied by one of

Table 3.1 Distribution of age at learning to walk in 114 affected boys

Age (months)	Number	Cumulative (%)
8–9	1	0.9
10–11	3	3.5
12–13	10	12.3
14–15	20	29.8
16–17	16	43.8
18–19	26	66.6
20–21	6	71.9
22–23	3	74.5
24–25	21	92.9
26–27	1	93.8
28–29	0	93.8
30–31	3	96.4
32–33	0	96.4
34–35	0	96.4
36	4	99.9

Table 3.2 Percentile distribution of age at apparent onset in 144 affected boys

Percentile	Age (years)
25	<2
50	2.4
60	2.8
70	3.4
75	3.7
80	4.1
90	5.1
95	6.1
99	7.8

the authors, percentiles were obtained by fitting the best curve to the data. It will be seen that, in 90% of cases, the onset is before school age (about 5 years). Despite our increased understanding of DMD and the improved diagnostic tools, a recent survey, published in 1999 by Bushby *et al.* in *The Lancet*, suggested that the mean age at diagnosis for DMD in the United Kingdom (UK) is

4 years 10 months, virtually identical to the age at diagnosis in the early 1980s. Twelve years later, a UK-wide report (Ricotti *et al.* 2013) indicated that the mean age at diagnosis was ~4.5 years. Interestingly, this coincides with the age at which children start school, and it is not uncommon for the child to be referred because the school teacher has noticed difficulties with physical education.

It is often stated that, if the parents have already had an affected son, the onset of the disorder in a second affected son is noted to be earlier, because they are conscious of the possibility. But this is by no means always true, as shown in Table 3.3 where the age at onset is given for affected brothers in cases where this information was personally recorded by one of the authors **at the time the diagnosis was confirmed** in each case.

Nevertheless, a recent report (Pane *et al.* 2013) reported that 45% of boys, with a mean age of 27 months, were found to have a suboptimal developmental quotient, with the motor function and speech and language domain more frequently affected after the age of 1 year, compared to their peers, suggesting that the early presentation might be secondary to the dysfunction of dystrophin in the brain, rather than the muscle. On close questioning, in almost all cases, the affected child **was never able to run or jump properly**. Other complaints at the time of onset included a waddling gait, walking unsteadily with a tendency to fall easily, toe walking, and difficulty in rising from the floor and climbing stairs. In a few instances, weakness was first noticed after the child had sustained a fracture following a fall. Sometimes, the parents noted a tendency to ‘throw out

Table 3.3 Age at onset in affected brothers

Case number	Age at onset (years)		
	Firstborn	Second-born	Third-born
11	2	7	–
90	5	3	–
100	2.5	5	–
111	2.5	4	–
121	1.75	1.5	–
528	1.5	6	–
587	4	2.5	–
593	7.5	7.5	–
1761	4.5	4.5	4.5
2009	3	1.5	–

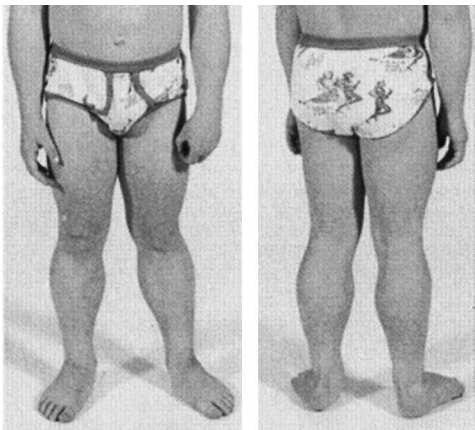


Fig. 3.1 A 4-year-old boy with DMD. Note the enlarged calves.

Table 3.4 Cases with delayed onset

Case number	Age (years)		Death	Comments
	Onset	Chairbound		
144	8	–	–	Now aged 10 and moderately affected
124	8	11	20	–
1741	8	12	–	Parents not good witnesses
2197	9	11	–	Severely mentally retarded (IQ <50)
112	9	12	21	–

his leg’ when walking, for the ‘feet to turn in’, or their attention was even drawn to the enlargement of the calf muscles (see Fig. 3.1).

Although most cases present in early childhood, occasionally, the diagnosis is not made until the age of 8 or 9. This is more often the case in children who have learning difficulties or other psychiatric co-morbidities (that is, autism; see Central nervous system, p. 106), which can induce professionals to attribute ‘clumsiness’ and other physical difficulties to the presumed primary psychiatric diagnosis. Even in these cases, the classical difficulties of early childhood can always be elicited retrospectively. This is confirmed by several ‘late’ presenters, assessed by one of the authors (Emery), and reported in Table 3.4.

Other presenting features

The most common additional feature at presentation is delayed intellectual milestones and, in particular, delayed speech development, affecting 50–70% of DMD children, noted at presentation. It is not uncommon that

concern related to the delay in speech precedes concerns related to the delayed motor development and muscle weakness. Although the majority of children eventually acquire the ability to speak normally, one-third of cases will have significant learning difficulty. Autistic features associated with severe mental retardation (see further text) can, not infrequently, be a reason for delayed diagnosis, because muscle weakness is masked. For this reason, checking the SCK in all boys presenting with developmental delay is essential.

Myoglobinuria and muscle cramps, mimicking a metabolic myopathy, are recognized associations in BMD and were considered rare in DMD. However, since the introduction of corticosteroid treatment for DMD, cramps and myoglobinuria are increasingly recognized as a feature (Garrood *et al.* 2008).

'Failure to thrive' may sometimes be an early feature of DMD. In keeping with previous observations, the authors have observed several children who presented in the first 18 months of life with unexplained failure to thrive. It is possible that this complication is secondary to the excessive muscle breakdown that is characteristic of these early phases of the disorder, although this remains speculative. It is important to consider DMD in the differential diagnosis of failure to thrive in boys where liver transaminases are raised and to check the SCK; otherwise, the child may undergo an unnecessary liver biopsy (McMillan *et al.* 2011).

Muscle pseudohypertrophy

The most obvious feature in the early stages of the disease is enlargement of the calf muscles, which are often said to feel 'firm' or 'woody'. Of 89 cases where the size of the calves was noted at some time in the course of the disease, in at least 85 (96%), they seemed much larger than normal. However, such enlargement may also involve the masseters, deltoids, serrati anterior, and quadriceps, and occasionally other muscles as well. Muscle enlargement is due, at least in part, to an excess of adipose and connective tissue, and therefore the term 'pseudohypertrophy' is widely used. But true (work) hypertrophy may also play a role in the early phase of the disease as a compensation for weakness in other muscles. However, in DMD, it is difficult to imagine this as being an important factor, because, in some cases, such muscle enlargement can be extensive (see Fig. 3.2). Enlargement of the tongue is a relatively frequent, but late, feature of the disease.

Interestingly, if an affected boy develops poliomyelitis, pseudohypertrophy is not present in an affected limb, suggesting that pseudohypertrophy requires an

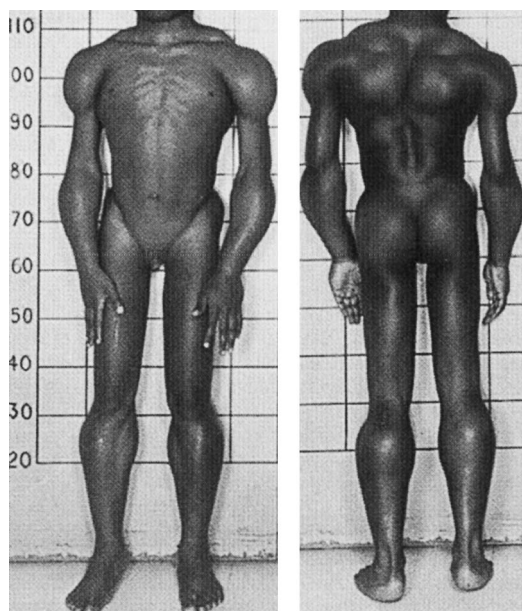


Fig. 3.2 Extensive muscle enlargement (pseudohypertrophy) in a case of DMD.

Reproduced courtesy of Dr Sarah Bunday.

intact nerve supply. Pseudohypertrophy is seen in other forms of muscular dystrophy, typically in sarcoglycanopathies and limb-girdle muscular dystrophy (LGMD2I), and even occasionally in the mild form of SMA.

Distribution of muscle weakness

Muscle involvement is always bilateral and symmetrical. In general, in the early stages of the disease, the lower limbs are affected more than the upper limbs, and the proximal muscles more than the distal muscles. At this stage, certain muscles are predominantly affected, including: latissimus dorsi, the sternocostal head of the pectoralis major, brachioradialis, biceps, triceps, iliopsoas, glutei, and quadriceps. The involvement is highly selective. For example, the quadriceps are more affected than the hamstrings, triceps more than biceps, wrist extensors more than flexors, neck flexors more than extensors, dorsiflexors of the feet more than the plantar flexors. Even within a single muscle, there is differential involvement. For example, the sternocostal head of the pectoralis major muscle is more affected than the clavicular head, but, in contrast, the clavicular head of the sternomastoid muscle is more affected

than the sternal head. Differential muscle involvement can be seen in many muscle disorders, including DMD. In some conditions, it can be a useful diagnostic tool; in DMD, however, its use is more likely to be as a useful outcome measure for clinical trials (Finanger *et al.* 2012; Schreiber *et al.* 1987). Differential muscle involvement in DMD becomes less clear as the disease progresses, so that ultimately such patterns are no longer obvious. Later, slight facial weakness often develops, and the intercostal muscles also become affected, followed by the involvement of the chewing and swallowing muscles. Sphincter control is never lost.

Early signs

This pattern of muscle involvement results in several well-defined physical features associated with the disease. Weakness of the gluteus medius and minimus muscles (which abduct the hip and hold the pelvic bone down to the greater trochanter of the femur) results in the pelvis tilting down toward the unsupported side when an affected child raises his leg from the ground (positive Trendelenburg sign). To compensate for this, he inclines toward the supporting leg. As he moves forward, this action is continually repeated and results in the broad-based, waddling gait that is so characteristic of DMD. But, as Professor Dubowitz (1995) has pointed out, ‘not everything that waddles is muscular dystrophy,’ for other conditions can also produce this type of gait (for example, SMA). Weakness of the gluteus maximus muscle (which powerfully extends the hip) results in a tendency for the pelvis to tilt forward, and, in order to compensate for this, a lumbar lordosis develops. In order to maintain his balance, and possibly because of an imbalance between the dorsiflexors and plantar flexors, the affected child also tends to walk on his toes.

Weakness of the knee and hip extensors results in the classical Gowers’ manoeuvre—the child climbs up his thighs in order to extend the hips and push up the trunk (see Fig. 3.3). However, it may be impossible to elicit this sign before the age of 4 or 5 years. Even before this age, we have found that an affected child is unable to rise from a sitting position on the floor if he is asked to **keep his arms folded** (which prevents him from pushing on his thighs or on the floor), whereas a normal child can accomplish this quite easily.

In the early stages of the disease, it may also be difficult to elicit weakness of the pectoral girdle musculature by formal testing. However, if the child is grasped around the chest from behind and an attempt made to lift him, there is a tendency to ‘slide through’ the examiner’s arms. Also, by placing the

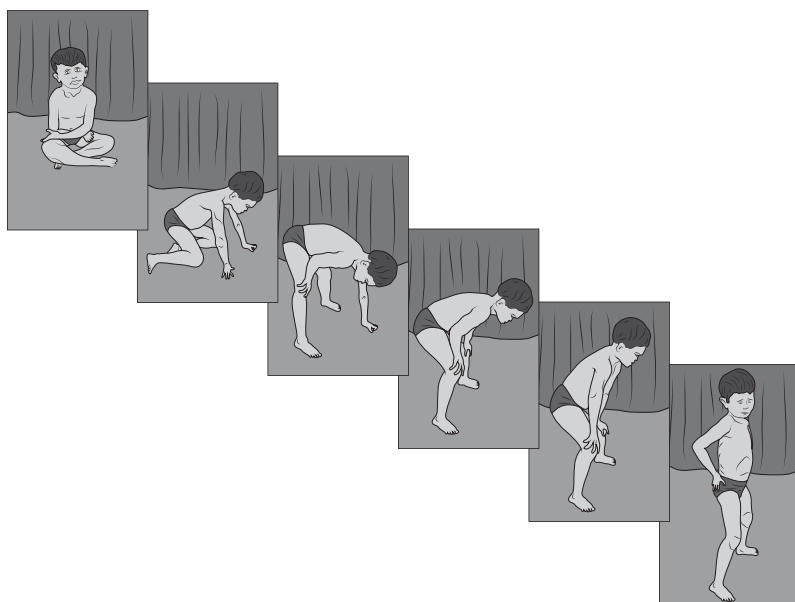


Fig. 3.3 A 5-year-old with DMD, showing the typical Gowers' manoeuvre whilst rising from the floor.

examiner's hands inside the upper arms, a normal child can be held up with comparative ease, but not an affected child. Both these signs are positive by the age of 4 years, and sometimes earlier. As the disease progresses, winging of the scapulae becomes apparent.

The affected muscles are not tender (as it occurs often in myositis), and there is no voluntary or percussion myotonia. As the muscles become weaker and wasted, the corresponding tendon reflexes become depressed, though good ankle jerks are retained for a long time and are the last of the tendon reflexes to disappear. The plantar responses are always flexor, and there is no sensory loss.

In the early stages of the disease, apart from the obvious difficulties of trying to keep up with their peers, affected boys usually make few complaints, apart from, occasionally, tenderness and stiffness following exercise, particularly in the calf muscles.

Another frequent complaint is toe walking, which becomes more evident when children are attempting to run or are tired. This is, at least in part, due to shortening of the heel cords (Achilles tendon). Most children with DMD have contractures of the Achilles tendon and, more rarely, of the hip flexors and ileo-tibial bands or hamstrings at presentation.

Progression

The weakness is progressive, but nevertheless the overall function can improve in the first few years following the diagnosis, due to improved coordination of children in that age range. It is because of this fluctuation that the assessment of the efficacy of suggested therapy has to take into account both the age and stage of the condition and needs to be evaluated over a prolonged period, often 12 months.

As the disease progresses, the lumbar lordosis becomes more exaggerated and can be a cause of low backache. The waddling gait becomes more noticeable, and shortening of the Achilles tendon becomes increasingly marked, and an equinovarus deformity develops, though this is more obvious when the boy becomes confined to a wheelchair. Respiratory muscles, although involved, to some extent, in all stages of the disorder, are never significantly weak in ambulant children, who will consistently have a forced vital capacity (FVC) of >70%.

As the condition progresses, to begin with, an affected boy may find he only needs a wheelchair at certain times (for example, when going outside), but inevitably he will become permanently confined to a wheelchair. The age at which this occurs is more precise and much better documented than the age at onset (see Table 3.5). In the series obtained by one of the authors (Emery) and presented in this table, there had been no intervention to prolong ambulation by

Table 3.5 Percentile distribution of age at becoming confined to a wheelchair in 120 affected boys not treated with corticosteroids

Percentile	Age (years)
10	6.7
20	7.4
30	7.8
40	8.2
50	8.5
60	8.9
70	9.3
75	9.6
80	10.0
90	11.0
95	11.9
99	13.2

Table 3.6 Age at death related to age at becoming confined to a wheelchair

	Age (years) confined to a wheelchair			
	≤7	8	9	10 or more
Number	9	18	15	13
Mean	15.65	16.52	17.44	18.15
SD	3.36	2.29	2.65	3.00
Number	27		28	
Mean	16.23		17.77	
SD	2.66		2.79	

pharmacological, orthopaedic, or orthotic measures, and therefore the data relate to the natural history of the disease.

Of 120 affected boys, in whom the age at becoming confined to a wheelchair was reliably known, in 95% of cases, this occurred by the age of 12 years. The age at becoming confined to a wheelchair was not significantly correlated with the age at onset but was significantly correlated with the age at death ($N = 55$; $r = 0.33$; $p < 0.02$). The difference in the mean age at death in boys who became wheelchair-dependent by 8 years of age ($N = 27$; mean, 16.23 years; standard deviation (SD), 2.66 years), compared with those who became wheelchair-dependent after 8 years of age ($N = 28$; mean, 17.77 years; SD, 2.79 years), is statistically significant ($p < 0.05$). It would seem that the age at death after 15, in boys who have not been treated with corticosteroids or non-invasive ventilation (NIV), increases roughly by 1 year for each year that a boy remains ambulant after the age of 7 up to the age of 10 or more (see Table 3.6). In general terms, the earlier a boy becomes confined to a wheelchair, the poorer the prognosis. More recent data, however, show the positive impact of corticosteroid administration in positively influencing the disease course; the systematic use of corticosteroids has, for example, shown that the mean age of loss of independent ambulation can be extended between the age of 12.5 and 14 years, depending on the regimen of corticosteroid use (Ricotti *et al.* 2013).

Assessment of motor ability

It is valuable to be able to chart the course of the disease in patients. A number of systems have been devised for doing this, which depend on assessing either the muscle strength or functional ability.

1 Muscle strength:

- Medical Research Council (MRC) grading (0–5);
- ergometry.

2 Functional ability:

- Swinyard grade (1–8);
- Vignos grade (1–10);
- Hammersmith motor ability score (0–40);
- North Star Ambulatory Assessment (NSAA) (0–34);
- 6-minute walk test;
- ‘CIDD’ grade for upper limbs (1–6);
- Egen Klassifikation (EK) scale for upper limbs (0–10).

Details of the various grading systems will be discussed in more detail later. A very detailed functional scoring system has also been developed by Cornelio *et al.* (1982), which is expressed as the sum of single scores for gait, climbing stairs, getting up from a chair, and getting up from a seated position on the floor.

Since there is inevitably a subjective element in such methods, in order to make comparisons, either between different patients or with the same patient over a period of time, they are best carried out by the same person. For many years, one of the authors (Emery) has used the Swinyard and Vignos grades of most patients examined (186 observations on 110 patients), and the results are given in Fig. 3.4, Fig. 3.5, and Fig. 3.6. Both grades correlate well with the progress of the disease. However, there is clearly considerable variation between different boys of the same age.

Measuring functional abilities with a reliable and easy-to-administer assessment tool has become increasingly important over the years. One scale developed in the 80s and 90s is the Hammersmith scale, in which a series of tasks are measured. Control children acquire the ability to perform all the assigned tasks by the age of 4, whilst DMD children almost inevitably score <40/40, even in the early phases of the disease (see Table 3.6). Loss of independent ambulation usually occurs with values $\leq 18/40$. As this scale also highlights, there is a considerable spread of functional abilities for DMD children at any given age. More recently, an adapted version of this scale, to address ceiling issues of the Hammersmith scale, was developed, the NSAA scale. This has become the scale of choice; it has been validated on 500 boys with DMD, as part of the UK North Star Network and in the Italian North Star Network, and is currently in use as one of the outcome measures in a number of clinical trials for DMD. This scale can be found in Table 3.7 and on the Registry of Outcome Measures at: <<http://www.researchchrom.com/>>.

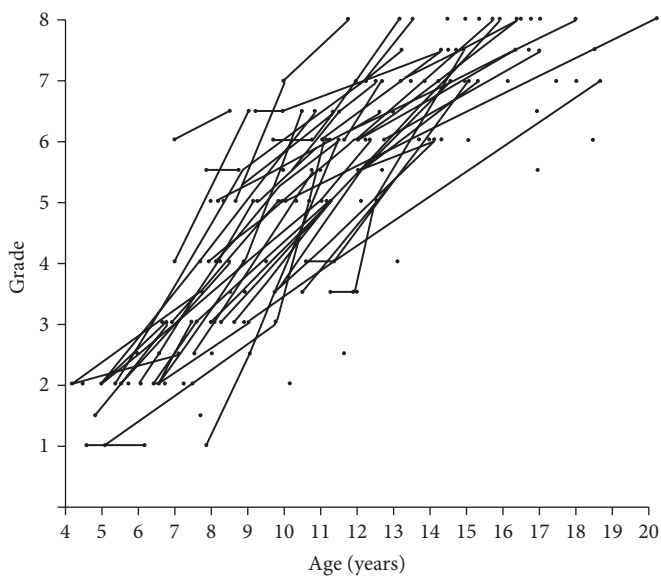


Fig. 3.4 Swinyard grade and age in boys with DMD. Points are joined for assessments on the same individual.

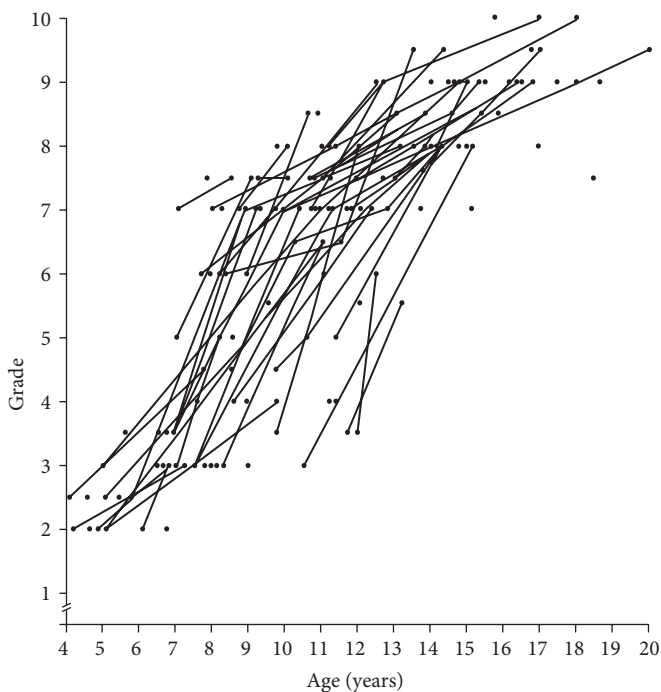


Fig. 3.5 Vignos grade and age in boys with DMD. Points are joined for assessments on the same individual.

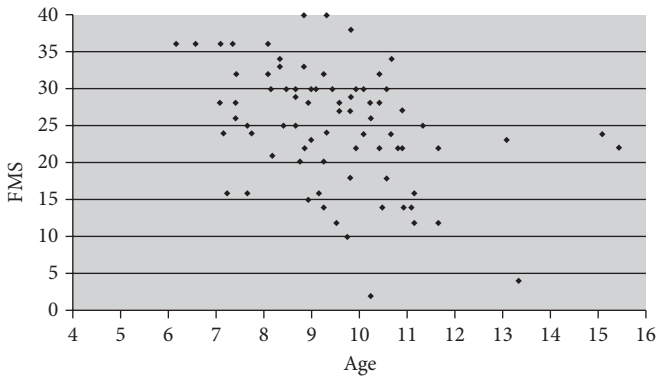


Fig. 3.6 Functional motor scale (FMS, Hammersmith Hospital motor scale) plotted against age in years in a cohort of children with DMD. Each point represents an assessment of a different child. The graph shows the progressive loss of function.

But, apart from variations in motor ability between boys of the same age, affected boys also differ in their general appearance. Some retain their subcutaneous fat and muscle bulk, whereas others become thin and atrophic. This is graphically demonstrated in the two boys of similar age, shown in Fig. 3.7. The reason for this is not clear, but there is a tendency for affected brothers to follow a similar pattern. In most cases, sexual development is normal, though puberty is delayed in a proportion of cases.

Later stages

As the disease progresses and muscle weakness becomes more profound, contractures increasingly develop, particularly flexion contractures of the elbows, knees, hamstrings, and hips. Later, movements of the shoulders and wrists also become limited. Talipes equinovarus deformity of the feet becomes marked, with the talus bone protruding prominently under the skin on the dorsum of the foot. Unless adequate support is provided in the wheelchair, and not infrequently despite the adequate support, a severe kyphoscoliosis develops. Thoracic deformity poses the most serious problem, as it restricts adequate pulmonary airflow on the compressed side (see Fig. 3.8).

The respiratory problems are also aggravated by weakness of the intercostal muscles. About halfway through the course of the disease, a gradual deterioration begins in the pulmonary function, with reduced maximal inspiratory and expiratory pressures. By the later stages, there is a significant reduction in the total lung capacity and an increase in the residual volume.

Table 3.7 North Star Ambulatory Assessment

Activity	2	1	0	Comments
1 Stands	Stands upright, still and symmetrically, without compensation (with heels flat and legs in neutral) for minimum count of 3 seconds	Stands still, but with some degree of compensation (for example, on toes, or with legs abducted, or with bottom stuck out) for minimum count of 3 seconds	Cannot stand still or independently, needs support (even minimal)	
2 Walks	Walks with heel-toe or flat-footed gait pattern	Persistent or habitual toe walker, unable to heel-toe consistently	Loss of independent ambulation—may use knee–ankle–foot orthoses (KAFOs) or walk short distances with assistance	
3 Stands up from chair	Keeping arms folded Starting position 90° hips and knees, feet on floor/supported on a box step	With help from thighs, or push on chair, or prone turn	Unable	
4 Stands on one leg—right	Able to stand in a relaxed manner (no fixation) for count of 3 seconds	Stands, but either momentarily or needs a lot of fixation, for example, by knees tightly adducted or other trick	Unable	
5 Stands on one leg—left	Able to stand in a relaxed manner (no fixation) for count of 3 seconds	Stands, but either momentarily or needs a lot of fixation, for example, by knees tightly adducted or other trick	Unable	
6 Climbs box step—right	Faces step—no support needed	Goes up sideways or needs support	Unable	
7 Climbs box step—left	Faces step—no support needed	Goes up sideways or needs support	Unable	
8 Descends box step—right	Faces forward, climbs down, controlling weight-bearing leg. No support needed	Sideways, skips down or needs support	Unable	

9 Descends box step—left	Faces forward, climbs down, controlling weight-bearing leg. No support needed	Sideways, skips down or needs support	Unable	
10 Gets to sitting	Starts in supine—may use one hand to assist	Self-assistance, for example, pulls on legs, or uses head on hands, or head flexed to floor	Unable	
11 Rises from floor	From supine—no evidence of Gowers' manoeuvre	Gowers' evident	(a) NEEDS to use external support object, for example, chair, OR (b) unable	Time (00.0 seconds)
12 Lifts head	In supine, head must be lifted in midline. Chin moves towards chest	Head is lifted, but through side flexion or with no neck flexion	Unable	
13 Stands on heels	Both feet at the same time, clearly standing on heels only (acceptable to move a few steps to keep balance) for count of 3 seconds	Flexes hip and only raises forefoot	Unable	
14 Jumps	Both feet at the same time, clear the ground simultaneously	One foot after the other (skips)	Unable	
15 Hops right leg	Clears forefoot and heel off floor	Able to bend knee and raise heel, no floor clearance	Unable	
16 Hops left leg	Clears forefoot and heel off floor	Able to bend knee and raise heel, no floor clearance	Unable	
17 Runs (10 metres)	Both feet off the ground (no double stance phase during running)	'Duchenne jog'	Walk	Time (00.0 seconds)
TOTAL = /34				

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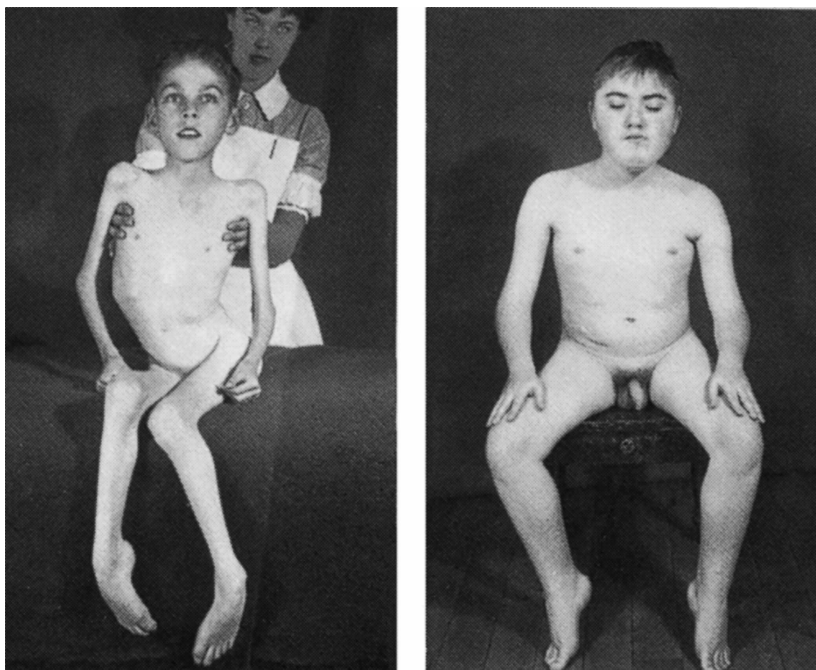


Fig 3.7 A 13-year-old boy (left) and a 12-year-old boy (right) with DMD.

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Fig. 3.8 Chest X-ray of an 18-year-old boy severely affected with DMD, showing gross thoracic deformity.

Whilst arterial oxygen tension (pO_2) and carbon dioxide tension (pCO_2) are normal, both during the day and night in ambulant children, with the progressive decrease of respiratory function, older teenage patients will slowly drift into respiratory failure; this usually does not occur when the FVC is $>50\%$ but is frequent for patients with an FVC of $<35\%$. As the intercostal muscles are less used during the night, especially during deep sleep, the exclusively diaphragmatic breathing is not sufficient to maintain normal gas exchange during the night. The first sign of respiratory failure is therefore night-time hypoventilation, with a fall in pO_2 and an accumulation of carbon dioxide, followed by rapid restoration of normal gas tension following arousal. This 'early respiratory failure' phase can last for several months, or even longer, but, when abnormal gas tensions occur during the day, the prognosis for long-term survival is poor. Patients with daytime hypercapnia are unlikely to survive >9.7 months (mean value) without the institution of NIV.

Cardiac muscle is also affected; serial cardiac echocardiographs demonstrate a progressive decline in the left ventricular function and will be discussed later. Premature death from cardiac failure may occur in about 15–20% of patients and has been seen by one of the authors (Quinlivan) in children as young as 11 and 12 years of age. Increased survival of young adults with DMD is now increasingly being associated with reports of sudden death, suggesting that the surveillance of the cardiac conduction system also needs to be performed regularly in the older patients. In the personal experience of one of the authors (Emery), from data collected between 1965 and 1985, there were 14 autopsy reports of DMD where the primary causes of death were given as pneumonia (11), 'respiratory failure' (1), diphtheria at age 8 (1), and acute cardiac arrhythmia (1). Carriers of DMD are also at increased risk of developing dilated cardiomyopathy.

That age at death might be, in some way, related to socio-economic factors is not borne out in the present series of patients. When information on social class was available, there was no apparent relationship with the age at death (see Table 3.8). However, a significant correlation has been shown to exist between DMD families and socio-economic deprivation (Bushby *et al.* 2001). Hospital attendance rates may be lower for children from impoverished backgrounds because of cost implications and potential financial hardship, especially if parents are required to take time off work; potentially this could affect prognosis. Lack of treatment compliance will almost certainly affect long-term prognosis.

The age at death was not significantly correlated with the age at onset, but, as we have seen, it was correlated with the age at becoming wheelchair-dependent. The percentile distribution for DMD individuals of the age at death in the year 1985, that is, before the introduction of corticosteroid treatment and NIV, is given in Table 3.9. In 90% of cases, this occurred before the age of 20.

Table 3.8 Age at death in DMD and social class of parents

Age at death (years)	Number in social class [†]					Total
	5	4	3	2	1	
<10	–	1	–	–	11	
10–14	1	1	4	2	–	8
15–19	4	15	11	5	1	36
20–24	2	–	1	–	–	3
Total	7	16	17	7	1	48
(χ ² = 10.92; <i>p</i> >0.05)						

[†] Social class is ranked from 5 (unskilled) to 1 (professional).

Table 3.9 Percentile distribution of age at death in 129 affected boys

Percentile	Age (years)
10	12.0
20	13.4
30	14.3
40	15.0
50	15.5
60	16.2
70	17.0
75	17.6
80	18.1
90	19.5
95	20.5
99	23.5

The considerable variation in the severity of the disease is further illustrated in Table 3.9, from which it can be seen that some boys may become wheelchair-dependent, or even die from cardiomyopathy, before the apparent onset in other boys.

Recent data from Eagle *et al.* (2002) suggest that intervention has changed the natural history of the disorder. Whilst the mean age at death in the Northern region was 14.3 years in the 1960s, this improved to 19 years in the 1990s for non-ventilated patients. This is due to improved management of patients, following the institution of a specialist neuromuscular centre providing

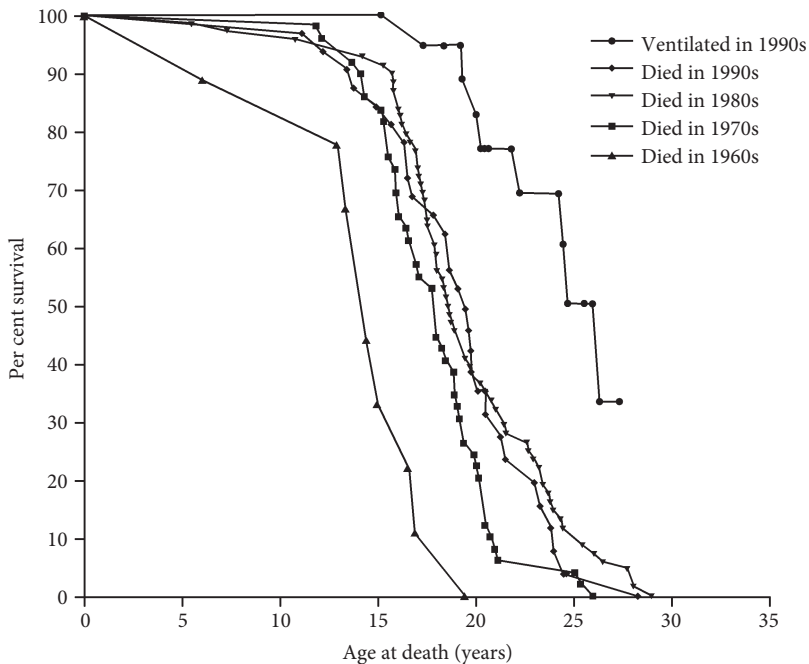


Fig. 3.9 Mean age of survival (years) of individuals with DMD. The mean age of survival for patients who do not develop an early and severe cardiomyopathy has recently shifted to over 26 years, according to a recent study of Eagles in the UK northern region.

Reproduced from *Neuromuscular Disorders*, Volume 12, Issue 10, Eagle, M., *et al.*, Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation, pp. 926–9, Copyright © 2002, with permission from Elsevier, <<http://www.sciencedirect.com/science/journal/09608966>>.

multidisciplinary care. This resulted, for example, in better management of scoliosis and intercurrent respiratory infections. Following the routine introduction of NIV in the later stages of the disease in the 1990s, the mean age of survival for those patients who did not develop early and severe cardiomyopathy increased to 24.3 years (Eagle *et al.* 2002) (see Fig. 3.9). More recent data from the same group showed continuing improvement in survival, with better outcomes for those who had scoliosis surgery and NIV, compared with those who were only offered NIV but not scoliosis surgery (Eagle *et al.* 2007). Similar research by Professor Anita Simonds in London indicated survival rates of 73% after 5 years in DMD patients treated with NIV (Simonds *et al.* 1998) and includes patients who have been able to complete their university studies and one who had even fathered a child. More recent data from Anita Simonds's group

Table 3.10 Number of boys with DMD with different ages at onset, becoming confined to a wheelchair, and death (Emery's series, up to 1985)

Age (years)	Number of boys with age at		
	Onset	Chairbound	Death
<2	24		
2	46		
3	27		
4	23		
5	14		
6	2	2	
7	3	15	
8	3	27	2
9	2	31	2
10		19	1
11		14	2
12		6	6
13		4	7
14		2	19
15			10
16			25
17			15
18			11
19			12
20			7
21			6
22			–
23			2
24			1
25			1
Total	144	120	129

suggest that the mean age of survival in the population of patients followed by her at Brompton Hospital is 29 years. Thus, the natural history of the condition is changing, with improved survival and some patients now surviving into their fourth and fifth decades; DMD can no longer be considered to be a pure childhood disease. In some countries, such as Denmark, there are more adults

living with DMD than children. In recent years, corticosteroid treatment has been shown to delay the loss of independent walking and preserve respiratory muscle function. The use of corticosteroids will be discussed in more detail later, but it seems likely that further improvement in survival will occur in years to come (see Table 3.10).

Disorders other than muscular dystrophy in affected boys

Several studies have shown that affected boys are of normal length and weight at birth, but subsequently growth is slower than normal, and later they are often of short stature. Otherwise, apart from learning difficulty and behavioural issues, such as autism or attention-deficit/hyperactivity disorder (ADHD), and problems directly relating to muscle weakness, most boys have very few health problems. The only other disorders recorded in the present series were recurrent urinary infections (2), left hydronephrosis with associated impaired renal function (1), unspecified congenital heart disease (1), undescended testes (1), and insulin-dependent diabetes mellitus (1). In the last case, the same disease also affected the boy's father. However, insulin resistance and hyperlipidaemia may become more prevalent with the newer cohort of older DMD survivors; this is because skeletal muscle plays an important signalling role in glucose and lipid homeostasis. Another recent development has been the description of several children who died suddenly from fat embolism, as a complication of DMD following minor trauma (McAdam *et al.* 2012). Whether this complication had been underrecognized in the past or is somehow related to the chronic use of corticosteroids and osteoporosis is currently not known.

Children with DMD usually have a larger head circumference, compared to their healthy siblings. This may not be true for those children who carry mutations affecting the expression of Dp71.

Summary and conclusions

The onset of DMD is insidious, but important hallmarks in the very early stages are frequent falls, a tiptoe gait, and an inability to run or jump properly. In over half of the cases, walking is delayed until at least 18 months of age. Manifestations of the disease are apparent in most cases before 5 years, but occasionally the diagnosis is delayed until the age of 8, or even 9 years of age. Some patients with the milder allelic variant BMD may also present in the first decade of life with similar, but less severe, symptoms. There are a few useful

clinical tips to help to differentiate DMD from BMD on clinical grounds. DMD children are not able to:

- ◆ run properly;
- ◆ jump off the ground;
- ◆ hop on one leg;
- ◆ lift their head off the bed;
- ◆ get up from a sitting position on the floor if asked to keep their arms folded.

Conversely, children affected with BMD are typically able to run, hop, lift the head off the bed, and get up from the floor without a Gowers' manoeuvre, at least at presentation.

Pseudohypertrophy of the calf muscles is present in almost all cases of DMD, and, in some instances, other muscles also show a pseudohypertrophic appearance. Wasting and weakness predominantly affects the proximal limb-girdle musculature, but, early on, muscle involvement is highly selective. A waddling gait, the Gowers' sign, and 'sliding through' the examiner's arms are useful diagnostic signs. However, even before the Gowers' sign can be elicited, affected boys are unable to rise from a sitting position on the floor if asked to keep their arms folded. The age at becoming wheelchair-dependent (which is invariably before the thirteenth birthday) is a prognostic sign in that the age at death after 15 increases roughly by 1 year for each year that a boy remains ambulant after the age of 7 up to the age of 10 or more.

Whilst 90% of boys died before the age of 20 until a decade ago, usually from respiratory problems, recent figures on individuals treated with NIV suggest that survival into the mid-late 20s can be achieved in a significant number of individuals, and, for some, survival is now extending into the fifth decade. However, there is considerable variation from one individual to another, and early cardiac involvement remains a negative prognostic factor. Further improvements in survival may be seen in years to come, as a consequence of corticosteroid treatment.

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Chapter 4

Confirmation of the diagnosis

Introduction to confirmation of the diagnosis

It is unlikely that an experienced paediatrician would have any difficulty in suspecting DMD in an otherwise healthy young boy who presents with a waddling gait, pseudohypertrophic calves, and a positive Gowers' sign. However, the diagnosis may not be so obvious in a very young child presenting with learning difficulty, or where a high creatine kinase (CK) has been found on routine blood testing, or where the presentation is later due to a relatively milder form of the disease. In these situations, it can be difficult to differentiate DMD from the allelic milder condition BMD. Because of the uniformly poor prognosis and the parents' need for reliable genetic counselling, it is essential that the diagnosis be firmly established as soon as possible. This depends on:

- 1 careful clinical examination;
- 2 determination of SCK;
- 3 muscle pathology;
- 4 genetic studies.

Serum creatine kinase

The enzyme CK (EC 2.7.3.2) catalyses the reversible transfer of a phosphate group from creatine phosphate to adenosine diphosphate (ADP), forming creatine and adenosine triphosphate (ATP).



The International Union of Biochemistry suggested the systematic name ATP: creatine phosphotransferase, with CK as the acceptable trivial name.

Over the years, a number of methods have been developed for measuring the activity of the enzyme, each depending on one of three approaches, details of which can be found in the second edition (1993) of the book.

In healthy infants, in the immediate newborn period, the level of activity of SCK is often somewhat raised to around 200–300 international units (IU). This may be due to muscular anoxia or the result of physical trauma. But, within a few days of birth, levels fall and are not very different from those of older

children. In young boys, there is no significant correlation with age, whilst, in adolescence, higher levels are not infrequent and may possibly be a reflection of increased muscle mass and physical activity in this period; otherwise, values are the same as in adults. The distribution of SCK levels in normal young healthy adult men is positively skewed, with a few individuals having high levels. But most values in young healthy adult men are <200 IU, which is a little higher than in women, presumably because the latter have less muscle mass.

Sibley and Lehninger in 1949 were the first to note that a serum enzyme (in this case, aldolase) could be raised in patients with muscular dystrophy. Some 10 years later, Ebashi and his colleagues in Tokyo (Ebashi *et al.* 1959) also showed that CK activity is raised in the serum of patients with muscular dystrophy, and this was confirmed the following year by Dreyfus *et al.* (1960) in Paris. Even at birth, and before the disease becomes clinically evident, SCK levels are considerably higher in boys with DMD than in normal boys, up to 100 times higher. However, as the disease progresses, levels fall but only approach normal values in the very late stages of the disease (see Fig. 4.1).

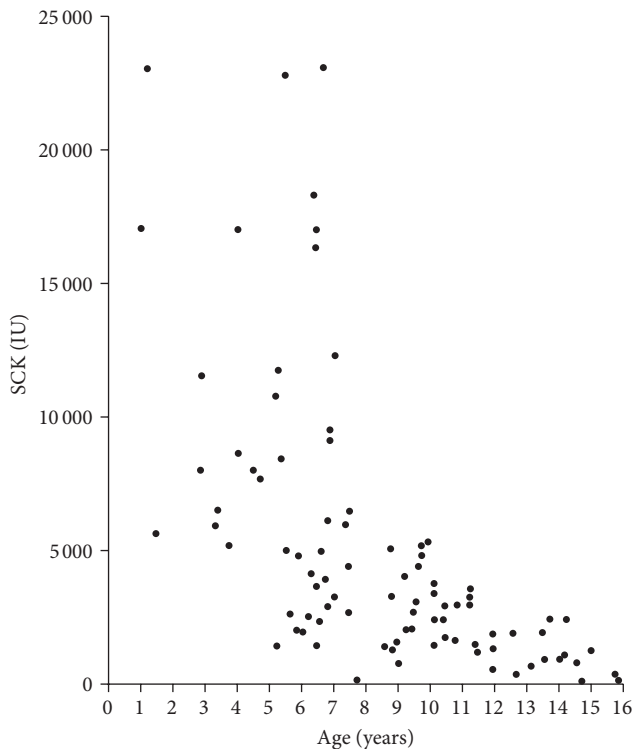


Fig. 4.1 SCK levels in boys with DMD.

The most likely explanation for the very high SCK levels in DMD is that the enzyme originates in muscle and escapes into the serum. The much lower levels in the later stages of the disease are, no doubt, due to the decrease in functioning muscle tissue and a reduction in physical activity. Levels certainly decrease, mostly around the time when affected boys become confined to a wheelchair.

Grossly elevated SCK levels (50–100 times normal) occur not only in DMD, but also in other muscular dystrophies presenting in childhood, e.g. BMD and some limb-girdle muscular dystrophies (LGMDs). Moderately elevated levels (up to ten times normal) occur in a wider range of conditions, including limb-girdle, facioscapulohumeral, and congenital muscular dystrophies, mild SMA, metabolic myopathies, and inflammatory myopathies. However, a young boy presenting with evidence of proximal muscle weakness, with a waddling gait, a positive Gowers' sign, and a grossly elevated SCK level, is most likely to have DMD or BMD, as these conditions are far more common than the others. Spontaneous myoglobinuria is rare, but we have observed it in young children with DMD in the first year of life or after corticosteroid treatment. Exercise-induced muscle cramps and myoglobinuria can be the presenting symptoms of BMD, which can often be mistaken for a metabolic myopathy such as McArdle disease.

Creatine kinase isoenzymes

CK exists in three molecular forms or isoenzymes. Each isoenzyme results from the dimeric association of two subunits, referred to as M and B, and the three isoenzymes are designated as MM, MB, and BB. The BB isoenzyme predominates in brain tissue, whereas the MM isoenzyme predominates in cardiac and skeletal muscle. The hybrid MB isoenzyme is a minor component in both cardiac and skeletal muscle. In normal serum, the isoenzyme is very largely MM, with only about 4% being MB. This small MB fraction, however, is significantly increased in patients with DMD or BMD. Though the MB form is found in cardiac muscle, its presence in serum in DMD is unrelated to cardiac involvement in the disease and presumably originates in dystrophic muscle in which there is more MB activity than in normal muscle. Bone also contains CK (BB isoenzyme). An increase in this isoenzyme has been reported in osteopetroses, bone tumours, and following fractures.

Other muscle enzymes that exist as isoenzymes and have been studied in detail in DMD are lactate dehydrogenase (LDH), aldolase, and pyruvate kinase (PK). LDH exists as five isoenzymes (LDH 1–5). In the serum of patients with DMD, there is a relative increase in the proportions of LDH 1–3, which reflects changes in the isoenzyme pattern in affected muscle tissue. There are muscle,

brain, and liver forms of aldolase, and the serum activity in DMD is predominantly that of the muscle type. PK exists as three isoenzymes (M2, M1, and L). In patients with DMD, the serum activity is mainly of the M1 type, which is the only PK isoenzyme found in the skeletal muscle, brain, and the major component in cardiac muscle. Finally, carbonic anhydrase III and β -enolase are skeletal muscle-specific enzymes, and these too are elevated in the serum of affected boys. Thus, in all these instances, the enzyme pattern in the serum of patients is similar to that in muscle tissue.

Other serum enzymes

Several other enzymes have been found to be raised in the serum in DMD, though none to the same extent as CK. The highest levels (10–20 times normal) occur with aldolase, PK, carbonic anhydrase III, and β -enolase. Less dramatic increases have been found in a number of other enzymes, including:

- ◆ LDH;
- ◆ phosphoglycerate mutase;
- ◆ alanine aminotransferase (glutamic–pyruvic transaminase, GPT);
- ◆ aspartate aminotransferase (glutamic–oxaloacetic transaminase, GOT);
- ◆ phosphohexose isomerase;
- ◆ phosphoglucomutase;
- ◆ α -hydroxybutyrate dehydrogenase;
- ◆ malate dehydrogenase.

Most of these are major ‘soluble’ (sarcoplasmic) muscle enzymes. Their increase in serum in DMD probably reflects an increased efflux through the muscle membrane, possibly augmented later in the disease process by the release from fibres undergoing necrosis. Evidence supporting this idea has been critically assembled by Rowland (1980), who also provides an extensive bibliography. The evidence may be summarized as follows.

- 1 The isoenzyme pattern of certain enzymes in serum closely resembles that of muscle tissue.
- 2 Under certain experimental conditions, it has been shown that enzymes are released from viable muscle tissue *in vitro*.
- 3 In patients, aldolase levels have been shown to be slightly higher in the venous return than in the corresponding arterial supply of the lower limb.
- 4 Almost all of the enzymes that are increased in the serum of patients are cytoplasmic, whereas enzymes that are bound in some way to intracellular structures are not found in the serum, using the commonly used detection methods.

- 5 When the activity of an enzyme is increased in the serum of patients with DMD, it is almost always decreased in the affected muscle, thus indicating that the enzyme originated from muscle.
- 6 The decline in serum enzyme levels, as the disease progresses, correlates well with the diminishing muscle mass.
- 7 Finally, the idea is further supported by the fact that, at least in some cases, the release is related to the molecular size. The molecular weights (MWs) of CK (81 000 daltons or 81 kDa) and aldolase (150 kDa) are considerably less than that of adenosine monophosphate (AMP) deaminase (320 kDa) and phosphofructokinase (400 kDa), which are also major muscle enzymes but which are virtually absent in serum of patients with DMD. However, some proteins with small MWs either do not appear at all in the serum of patients (adenylate kinase, 21 kDa) or only in relatively small amounts (myoglobin, 17 kDa).

The situation is therefore not simple and cannot be explained purely in terms of leakage from affected muscle. The serum level of an enzyme will be affected by its clearance rate, and its efflux from muscle will depend on its relative concentration in this tissue, its binding to intracellular structures, and possibly some form of selective force at the level of the muscle membrane. Recently, a link between the lack of dystrophin and a general dysregulation of vesicle trafficking was established in muscle cells. These authors suggested a disturbance of the export of proteins through vesicles which occurs independently and concurrently with the muscle necrosis (Duguez *et al.* 2013).

From a practical point of view, it is not possible to distinguish DMD and BMD on the basis of serum levels of various enzymes.

Muscle pathology

A great deal has been written about the pathology of muscle in various neuromuscular disorders. Several extensive monographs have been published that deal in detail with the various changes observed in the course of these diseases (Anderson 2002; Dubowitz 1985; Kakulas and Adams 1985). Here, only a brief description will be given of those changes in muscular dystrophy that are helpful in establishing the diagnosis.

Biopsy technique

Whilst, in the past, a lot of emphasis was put on the need to choose a moderately affected muscle, nowadays the availability of a direct protein test (the expression of dystrophin) allows a confident diagnosis to be made of DMD or makes it possible to differentiate it from other forms of dystrophy, even in muscles that are pathologically only minimally affected. The choice of the muscle biopsy is

therefore only determined by which muscle is safe and easy to access—this is usually the quadriceps.

Muscle biopsies can be obtained, either using an open technique or by a percutaneous needle; the latter leaves a smaller scar and can yield sufficient tissue for analysis. An open muscle biopsy will yield a larger sample which can allow more extensive analysis, particularly enzyme quantification, which is, however, not necessary if DMD is suspected. Excellent results are obtained with muscle frozen in liquid nitrogen-cooled isopentane, and studies made on cryostat sections. The latter procedure is associated with fewer artefacts and has the advantage that histochemical and immunohistochemical studies can be carried out on the material. Transverse sections are more informative than longitudinal

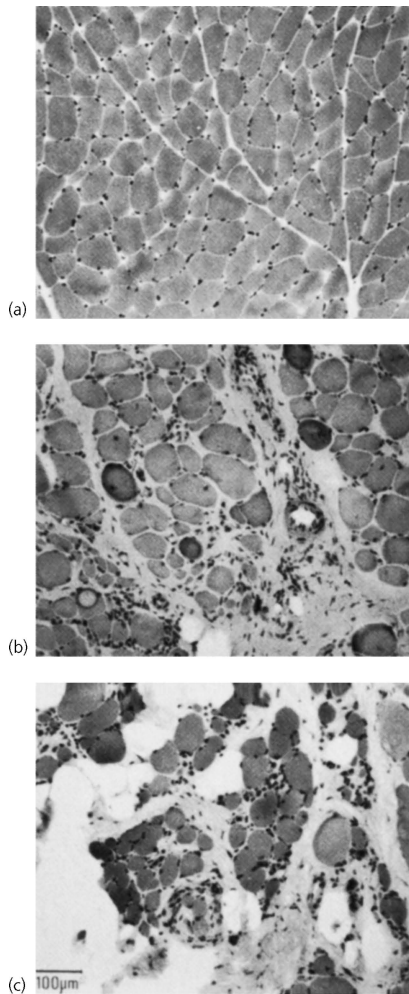


Fig. 4.2 Transverse cryostat sections of the gastrocnemius muscle from: (a) a healthy boy; (b) an early case of DMD; (c) an advanced case of DMD (haematoxylin and eosin).

sections, as they allow a better assessment of the variability in fibre size, internal nucleation, fibrosis, and degeneration and regeneration. Paraffin embedding of fixed material is no longer necessary, and this is an advantage because of the smaller quantity of muscle that can be obtained with a needle, compared to that obtained with an open biopsy.

In a well-established case of DMD, the changes observed in, say, the quadriceps or gastrocnemius muscles include an increased variation in fibre size, fibre necrosis with phagocytosis, and eventually replacement by fat and connective tissue (see Fig. 4.2). It is worthwhile considering some of these changes in a little more detail and tracing their development during the course of the disease.

Preclinical stage

Before there are any obvious clinical manifestations of the disease, there are already significant abnormalities in muscle pathology. Very early on, the only significant abnormalities may be an increased variation in fibre size and an increase in the number of prominent rounded fibres staining more densely with eosin—here referred to as eosinophilic fibres. In normal muscle, these fibres are absent or very infrequent, and, when present, they typically occur at the periphery of sections, indicating that they are artefactual.

On the other hand, in DMD, they are seen throughout sections and, in some cases, can be particularly frequent (see Fig. 4.3). These same fibres contain increased intracellular calcium, as revealed by histochemical staining with alizarin red S or a fluorescent method with pentahydroxyflavone (Morin) (see Fig. 4.4).

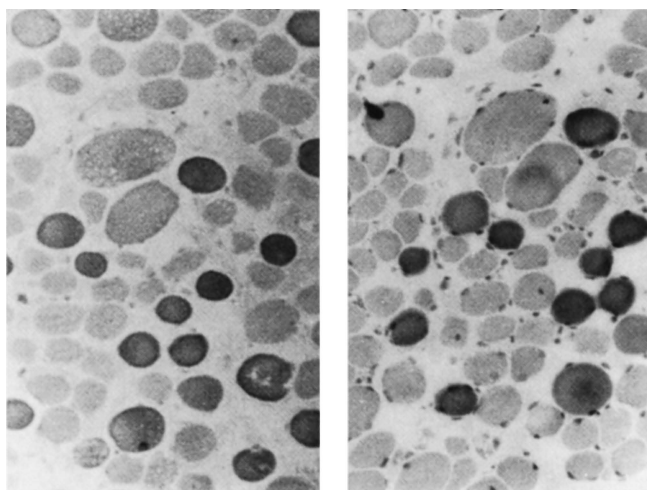


Fig. 4.3 Serial sections of the gastrocnemius muscle in a preclinical (2-year-old) case of DMD stained with (left) haematoxylin and eosin and (right) alizarin red S. Note the numerous eosinophilic/calcium-positive fibres, but no evidence of muscle fibre necrosis.

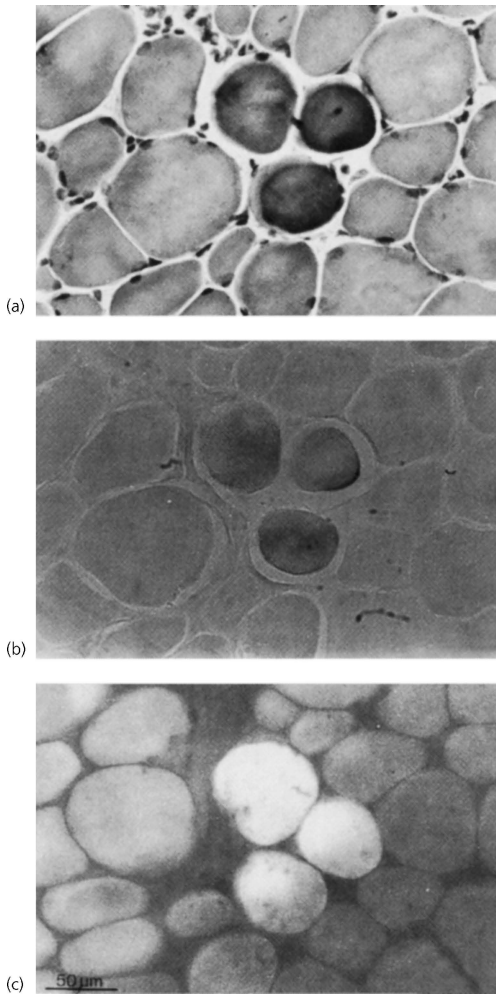


Fig. 4.4 Serial sections of muscle from an early case of DMD stained with: (a) haematoxylin and eosin; (b) alizarin red S; (c) fluorescent Morin.

Reproduced from Emery, A. E. H. and Burt, D., Intracellular calcium and pathogenesis and antenatal diagnosis of Duchenne muscular dystrophy, *British Medical Journal*, Volume 280, Issue 6211, pp. 355–7, Copyright © 1980, with permission from the author.

Increased intracellular calcium in skeletal muscle in DMD has been demonstrated not only histochemically, but also by X-ray microanalysis and chemical methods. The relevance of increased intracellular calcium in the pathogenesis of DMD will be discussed in Chapter 10.

The proportion of calcium-positive fibres in DMD is very variable, and the highest proportion has been found in a preclinical case (see Table 4.1). A significant increase in calcium-positive fibres also occurs in BMD, but to a much lesser degree in other muscular dystrophies. The intracellular accumulation of calcium appears to be the prelude to the breakdown and death (necrosis) of the muscle fibre.

Table 4.1 Proportion (%) of eosinophilic and calcium-positive fibres in cryostat sections of gastrocnemius muscle biopsy samples from boys with no neuromuscular disorder and boys with DMD

Age (years)		Proportion (%) in cryostat sections of	
		Eosinophilic fibres	Calcium-positive fibres
Controls (<i>N</i> = 7)			
5–12		<0.2	<0.1
DMD patients			
B103	10	6.0	5.8
B110	9	1.4	1.8
B115	7	3.0	2.9
B111	6	2.7	4.4
B106	5	4.1	6.5
B117*	3	3.7	53
B159*	2	15.3	18.3

* Preclinical cases.

Source: data from personal observation by one of the authors.

At this early stage in the disease process, regenerating fibres are also commonly found. They are recognized by their smaller size in cross-section, basophilic cytoplasm, high concentration of ribonucleic acid (RNA), and large, pale vesicular nuclei with prominent nucleoli. Other early changes are represented by focal areas of degeneration, with clusters of fibres undergoing degeneration/regeneration, at times affecting entire fascicles, and an increase in rounded fibres that are often hypercontracted. However, the process of regeneration becomes less frequent as the disease progresses, and fibres undergoing necrosis become more obvious. For reasons that are not yet clear, muscle fibres of smaller diameter seem more resistant to necrosis.

Later stages

The changes that take place as a muscle fibre undergoes necrosis are complex. As the intracellular structures are destroyed, the fibre is invaded by phagocytic cells. Often, one can observe localized necrosis and the degeneration of fibres, segmentally invaded by phagocytic cells. Muscle fibres in various stages of degeneration and regeneration are illustrated in Fig. 4.5.

As the necrotic fibres are phagocytosed, they are replaced by fat and connective tissue so that eventually only small islands of muscle tissue remain. There is a significant pathological overlap between DMD and BMD, and a confident

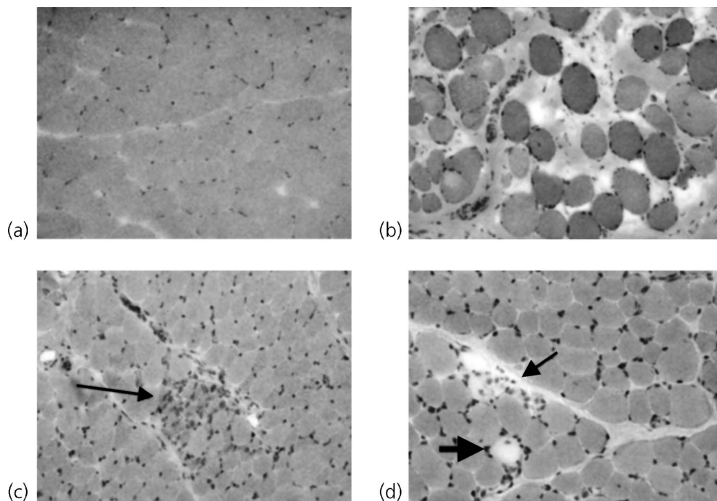


Fig. 4.5 (a) Normal muscle. (b) Duchenne muscle with hypercontracted fibres, abundant connective tissue, fat, and internal nuclei. (c) Duchenne muscle with cluster of basophilic regenerating fibres (arrow) and only mild fibrosis. (d) Duchenne muscle, showing a small cluster of necrotic fibres (small arrows) and an isolated one (big arrow).

pathological diagnosis must rely on studies of dystrophin expression, and not on simple morphological grounds.

The histological changes in muscular dystrophy are very different from those in neurogenic conditions such as SMA. In the latter group of disorders, all those muscle fibres associated with the defective neuron gradually atrophy. This produces the classical picture of group atrophy and is pathognomonic of SMA (see Fig. 4.6). The atrophy may be so profound as to present the appearance of ‘nuclear clumps’. In SMA, affected fibres undergo atrophy and tend to be grouped together. In contrast, in muscular dystrophy, affected fibres undergo structural changes, and these occur in individual fibres at random. However, especially in the more chronic forms of neurogenic atrophy, muscle fibres adjacent to groups of atrophic fibres may undergo changes similar to those seen in muscular dystrophy: variation in fibre size, central nuclei, and even occasionally fibre necrosis and phagocytosis. It is possible that such changes result from faulty attempts at reinnervation—the metabolism of these abnormally innervated fibres is disturbed in some way that then results in the structural changes that might resemble a muscular dystrophy. Usually, however, significant fibrosis is not observed in neurogenic disorders. On the other hand, it is not uncommon to observe ‘pseudoneurogenic’ changes in the muscle biopsy of some of the chronic forms of muscular dystrophy. Whilst this does not occur in DMD, it

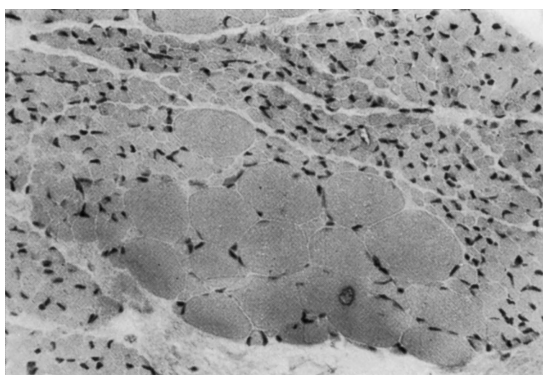


Fig. 4.6 Muscle fibre atrophy in SMA (Werdnig–Hoffmann disease). Note large groups of atrophic fibres (haematoxylin and eosin).

can be occasionally found in BMD and, more frequently, in facioscapulo-humeral and Emery–Dreifuss muscular dystrophies. Various investigators have pointed out the problems that can be encountered in the interpretation of the pathology. For example:

... small, angular fibers can derive from fiber splitting, which can be the result of either a chronic myopathy, denervation, or tenotomy. Small groups of atrophic fibers can result from splitting or regeneration after necrosis. The changes described as characteristic of myopathy have been reported in biopsies from muscles affected by chronic denervation. (Bradley and Fulthorpe 1978)

Dermatomyositis is an inflammatory myopathy that can affect children. The muscle biopsy can also show muscle fibre necrosis and regeneration, but the distinctive pathological features in this disorder are the lack of hypertrophic fibres and infiltration of muscle tissue by inflammatory cells (mainly lymphocytes and plasma cells). The latter is usually focal, around blood vessels, and within muscle fibres. However, the distinction between groups of phagocytic cells, as seen in DMD, and perivascular infiltrates of lymphocytes and plasma cells in polymyositis is not always clear. Interestingly, the autosomal dominant form of Emery–Dreifuss muscular dystrophy and LGMD2B can be mistaken for dermatomyositis, due to the amount of inflammation seen in the muscle biopsy. On the other hand, in some cases of childhood-onset dermatomyositis, muscle histology may show only minimal changes, or practically no significant changes at all; in this situation, muscle magnetic resonance imaging (MRI) can be very useful in differentiating muscle oedema, caused by inflammation, from fatty infiltration seen in the muscular dystrophies.

The finding that human leucocyte antigen (HLA) class I is significantly up-regulated at the sarcolemma in inflammatory myopathies, but not in mature muscle fibres in DMD or other muscular dystrophies, has provided an additional and useful tool in the differential diagnosis. In particular, upregulation of HLA class I at the sarcolemma may be the only change observed in inflammatory myopathies, even in the absence of other histological changes. The mainstay of the pathological diagnosis of DMD is, however, the muscle immunohistochemistry and/or Western blot analysis.

Muscle histochemistry

On the basis of physiological experiments in animals, and biochemical and histochemical studies on muscle tissue, it is possible to classify human skeletal muscle fibres into three distinct types, designated as 1, 2A, and 2B. Some of the differences between these fibre types are summarized in Table 4.2.

Animal experiments have shown that muscle fibres innervated by a single motor neuron possess similar physiological properties and identical histochemical characteristics. Thus, there would appear to be at least three types of motor neurons. Fig. 4.7 shows the effects of denervation, as it occurs in SMA. If this is rapidly progressive, there may be little time for reinnervation. However, if reinnervation occurs, from nerve fibres from another neuron, this will lead eventually to groups of atrophic fibres being of the same histochemical type.

The appearance of fibre-type atrophy in a case of Werdnig–Hoffmann disease is shown in Fig. 4.8, in which type 2 fibres are predominantly affected. However, both fibre types can atrophy after denervation, though most larger fibres tend to be of type 1. In muscular dystrophy and dermatomyositis, no particular fibre type is predominantly affected, and there is no grouping of fibre types.

Table 4.2 Some characteristics of various muscle fibre types in human skeletal muscle

	Type 1	Type 2A	Type 2B
Speed of contraction	Slow	Intermediate	Fast
Appearance (myoglobin content)	Dark	Dark	Pale
Size	Small	Intermediate	Large
Enzyme activities			
ATPase pH 9.4	+	+++	+++
ATPase pH 4.3	+++	+	+
Oxidative*	+++	++	+
Phosphorylase	+	++	+++

* NADH-tetrazolium reductase, succinic dehydrogenase.

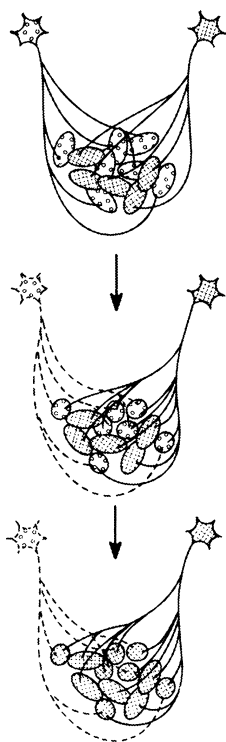


Fig. 4.7 Diagrammatic representation of the possible effects of denervation followed by reinnervation. For simplicity, only two types of fibres are illustrated.

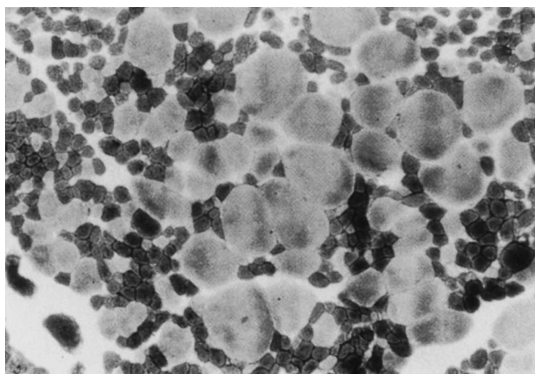


Fig. 4.8 Fibre-type atrophy in a case of Werdnig–Hoffmann disease (ATPase pH 9.4).

The reason why certain groups of muscles are especially affected early in the course of muscular dystrophy is not at all clear. This presumably reflects some difference in their biochemical/physiological properties, compared with muscles affected to a lesser degree and only in the later stages of the disease. Initial studies focused on the possibility that the selective involvement might be related to the

different proportions of type 1 and type 2 fibres of different muscles. Johnson *et al.* (1973) in particular correlated the proportions of type 1 and type 2 fibres in normal skeletal muscles that in DMD are either severely affected or relatively unaffected. They found that there was a tendency for the proportion of type 2 fibres to be higher in muscles that are more severely affected, but there was no simple correlation between fibre-type composition of individual muscles and their being affected or spared in the disease. Using their extensive data, we calculated there would appear to be a slight excess of type 2 (or deficiency of type 1) fibres in those muscles affected early in the disease, but there is considerable variation, and the difference from those muscles affected later in the course of the disease is not statistically significant (see Table 4.3). Whatever may be responsible for the differential involvement of muscles in DMD, it is not a simple matter of histochemical fibre type. Muscle MRI studies has, more recently, confirmed the gradient of skeletal muscle involvement in the legs of ambulant DMD boys, and interestingly also the rate of progression of changes over a 9-month period. The most significant changes were noted in the biceps femoris long head, vastus lateralis, and rectus femoris, whilst the biceps femoris short head and gluteus maximus progressed more slowly (Hollingsworth *et al.* 2013).

Table 4.3 Mean proportion (%) of type 1 and type 2 fibres in various normal human muscles (Johnson *et al.* 1973), which the present authors have divided into those muscles clinically affected early or late in the course of DMD

Proportion (%) of fibres in normal human muscles		
	Type 1	Type 2
Affected early		
Sternomastoid	35.2	64.8
Pectoralis major (sternocostal)	43.1	56.9
Triceps		
Surface	32.5	67.5
Deep	32.7	67.3
Brachioradialis	39.8	60.2
Extensor digitorum	47.3	52.7
Extensor digitorum brevis	45.3	54.7
Latissimus dorsi	50.5	49.5
Iliopsoas	49.2	50.8
Gluteus maximus	52.4	47.6
Vastus medialis		
Surface	43.7	56.3
Deep	61.5	38.5

Table 4.3 (continued) Mean proportion (%) of type 1 and type 2 fibres in various normal human muscles (Johnson *et al.* 1973), which the present authors have divided into those muscles clinically affected early or late in the course of DMD

Proportion (%) of fibres in normal human muscles		
	Type 1	Type 2
Affected early		
Rectus femoris		
Lateral head, surface	29.5	70.5
Lateral head, deep	42.0	58.0
Medial head	42.8	57.2
Tibialis anterior		
Surface	73.4	26.6
Deep	72.7	27.3
Mean	46.68	53.32
SD	–	12.74
Affected later		
Trapezius		
	53.7	46.3
Pectoralis major		
Clavicular	42.3	57.7
Biceps brachii		
Surface	42.3	57.7
Deep	50.5	49.5
Biceps femoris		
	66.9	33.1
Flexor digitorum brevis		
	44.5	55.5
Flexor digitorum profundis		
	47.3	52.7
Gastrocnemius		
Lateral head, surface	43.5	56.5
Lateral head, deep	50.3	49.7
Medial head	50.8	49.2
Soleus		
Surface	86.4	13.6
Deep	89.0	11.0
Mean	55.63	44.37
SD	–	16.42

Source: data from *Journal of the Neurological Sciences*, Volume 18, Issue 1, Johnson, M. A., *et al.*, Data on the distribution of fibre types in thirty-six human muscles—an autopsy study, pp. 111–29, Copyright © 1983.

Muscle innervation

In addition to the indirect changes, such as fibre-type grouping, a useful technique for studying denervated muscle is the intravital or supravital staining of motor nerve filaments and end-plates with methylene blue. The motor end-plate is a complex structure at the site where excitation is transmitted from the motor nerve to the muscle fibre (see Fig. 4.9). In SMA, there is branching of subterminal intramuscular nerve fibres (see Fig. 4.10), with collateral reinnervation and degeneration of motor end-plates.

Branching of nerve fibres in this way is found in all forms of SMA but is very rarely seen in normal or dystrophic muscle.

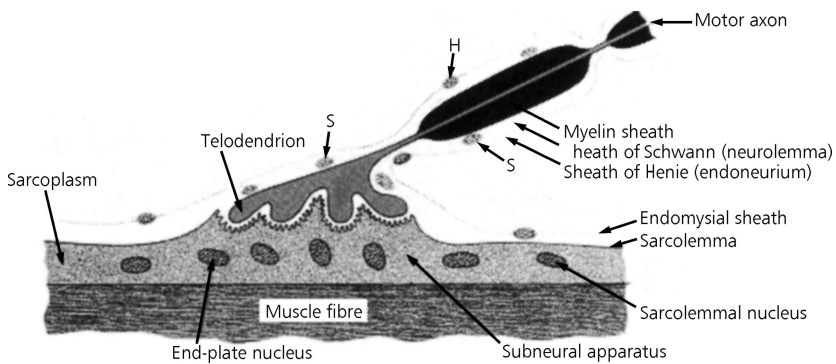


Fig. 4.9 The structure of the normal human end-plate. H, nucleus of the sheath of Henle; S, nucleus of the sheath of Schwann.

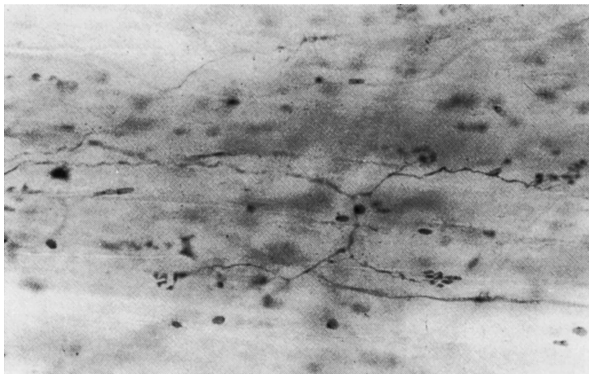


Fig. 4.10 Branching of subterminal intramuscular nerve fibres in a case of Wohlfart-Kugelberg-Welander SMA (supravital staining with methylene blue).

Electron microscopy

Electron microscopy of muscle has provided details of the ultrastructural changes that take place in muscular dystrophy. These include distension of the sarcoplasmic reticulum, Z-band degeneration ('streaming'), and disruption and loss of myofilaments, followed later by a complete disarray of the band structure (see Fig. 4.11).

These changes, however, are not specific to DMD but also occur in other forms of dystrophy. Various alterations in the sarcolemma have also been described early in the course of the disease and include defects in the plasma membrane and duplication of the basement membrane. These latter changes are common to conditions characterized by extensive degeneration and regeneration.

Role of genetic analysis of the dystrophin gene and of muscle biopsy

The approach to the diagnosis of DMD and BMD has been revolutionized by the introduction of gene markers and dystrophin studies. In fact, some have

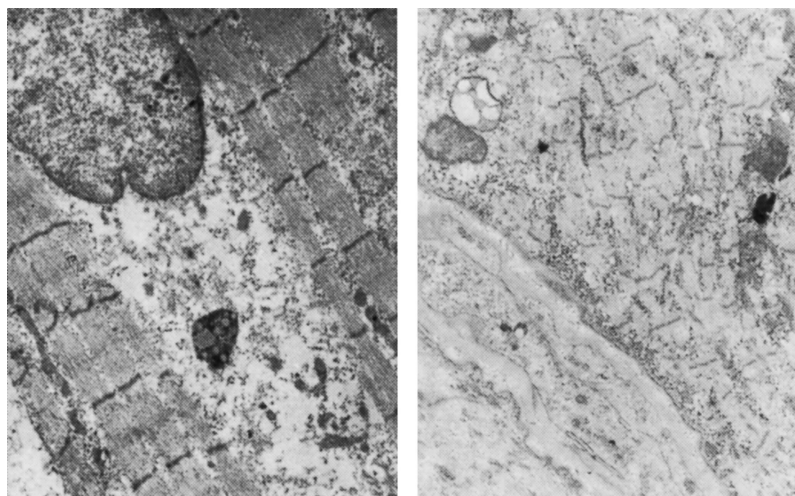


Fig. 4.11 Electron microscopy of skeletal muscle. (Left) Relatively early stage of DMD. The myofibrils have lost some myofilaments, with widening of the intermyofibrillar space which contains a lysosome (phosphotungstic acid, $\times 14\,000$). (Right) Later stage, showing disorganization of the band structure (2% uranyl acetate and lead citrate, $\times 7000$).

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argued that there is no place for any diagnostic investigations, other than an SCK test (because of its simplicity, cheapness, and relative specificity) and molecular genetic analysis of the dystrophin gene. Whilst, in many instances, this is the preferred path to establish the diagnosis, especially in cases where the clinical manifestations are already relatively advanced and the severity of the clinical phenotype unequivocal, a note of caution is necessary when interpreting data on presymptomatic young children. Indeed, as will become clear later, it is not always possible to predict with precision the phenotype resulting from mutations in the dystrophin gene by DNA analysis alone. The diagnostic approach that we recommend, in order to obtain a robust diagnosis for this severe, life-limiting condition, especially in young asymptomatic or paucisymptomatic children, is therefore that of performing both a muscle biopsy for direct dystrophin studies, as well as molecular genetic investigations to identify the primary genetic defect.

DNA studies on peripheral blood

As will be discussed in greater detail later, the gene locus for DMD and BMD has been located on the short arm of the X chromosome (at Xp21). The mutation detection technique most commonly used in the first decade, following the discovery of the dystrophin gene, was the Southern blot, i.e. the transfer of DNA after digestion with restriction enzymes, followed by hybridization with fragments of relevant genomic DNA (probes). With this technique, it is possible to follow the inheritance of the so-called restriction fragment length polymorphisms (RFLPs), which can be either close to the gene (extragenic) or actually within the gene (intragenic). In affected families, such linked RFLPs can therefore be used for tracking down the mutated chromosome X in a family by simply studying DNA obtained from peripheral blood (linkage analysis).

Unfortunately, because the gene is so large (approximately 3 megabases (mb)), not unexpectedly, even markers within the gene may show recombination (cross-over) with a particular mutation and could therefore lead to misdiagnosis. It has been shown, for example, that polymorphic markers that lie at the two extremities of the dystrophin locus show a recombination frequency of 0.12 or 12%. Using extragenic markers, the error rate can be even greater. Therefore, this has to be taken into account when using linked markers for diagnosis, and the use of informative flanking markers is therefore recommended (see Chapter 11).

Another approach that does not require other affected members in the family, in order to determine the so-called phase (see Chapter 11) of a DNA marker, is to use a gene-specific (complementary DNA, cDNA) probe. By using intragenic dystrophin probes, it is possible to determine if there is a **gene deletion or**

duplication. Indeed, roughly 70% of cases have a deletion or a duplication that can be detected on a Southern blot in this way.

An interesting finding is that deletions tend to be clustered around two 'hot-spot' regions (see Fig. 4.12). Thus, the majority of deletions can be detected by examining only a subset of exons within the gene. In the late 1980s, Jeff Chamberlain and his colleagues (1988) and Alan Beggs *et al.* (1990) developed a method, referred to as the 'multiplex method', in which a number of regions (which are deletion-susceptible) are simultaneously analysed by amplifying these regions using the polymerase chain reaction (PCR). This important technique involves amplifying very small amounts of DNA such that eventually

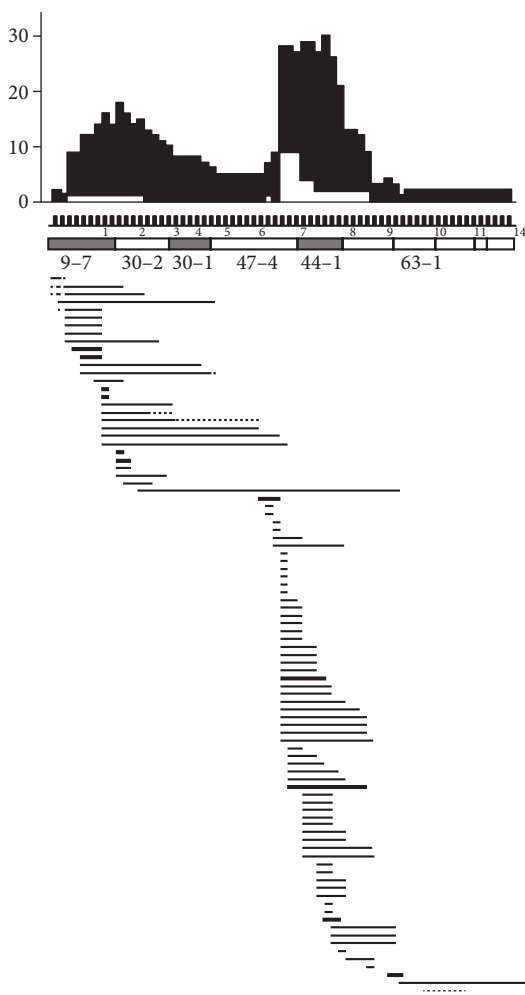


Fig. 4.12 Distribution and extent of gene deletions and duplications in DMD (solid) and BMD (open).

Reproduced from Bakker, E., Carrier detection and prenatal diagnosis of Duchenne/Becker muscular dystrophy by DNA-analysis, in C. S. Bartsocas (Eds.), *Genetics of neuromuscular disorders*, pp. 51–67, Alan R. Liss Inc., New York, USA, Copyright © 1989, with permission from John Wiley and Sons Inc.

there is sufficient to visualize on a gel by fluorescence when stained with ethidium bromide. Particular regions of the DNA (in this case, exonic regions most likely to be deleted) are amplified by PCR, using primers that specifically flank these regions.

A diagrammatic example of the multiplex method is shown in Fig. 4.13. In family 1, the affected boy (B) has a deletion of exon *f* (as does a fetus (F) at risk in the same family); in family 2, the deletion is more extensive and involves exons *a* to *e* inclusive; in family 3, there is a deletion of exon *a* only. This is an extremely rapid method for screening for deletions, and, by using two mixtures of primers, each of which amplifies nine exons, over 98% of detectable deletions can be identified in this way. Since the introduction of multiplex PCR, this technique has proved to be a rapid, reliable, and accurate method for detecting deletions. It requires only one day for analysis; with experience, it is easy to perform, and, unlike Southern blot analysis, it does not require radioactive probes. The method, of course, cannot be used, unless the patient being tested is related to an individual already known to carry a deletion detectable by the system.

If, as in family 4 in Fig. 4.13, there is no detectable deletion with this method, then resort has to be made to Southern blot analysis with full-length cDNA probes. The use of an automated and quantitative fluorescent PCR system has recently facilitated the detection of deletions, making it possible to assign the carrier status of females at risk. Moreover, more precise quantitation also allows

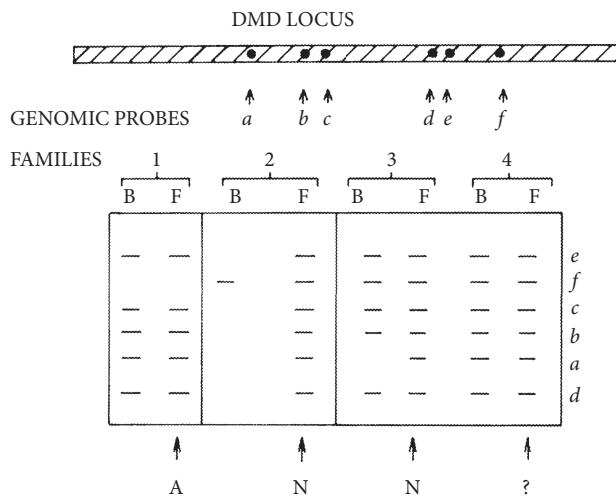


Fig. 4.13 Diagram of the results of the multiplex method used to simultaneously detect deletions in exons *a* to *f* in four families. B, boy; F, fetus; A, affected; N, normal.

the detection of duplications that were probably overlooked in the past using other techniques. Recent figures suggest that as many as 15% of children carry a duplication of part of the gene.

Regarding the genomic approaches proposed to detect mutation using genomic DNA, Mendell and colleagues (2001) introduced a sensitive modification of the single-strand conformation polymorphism (SSCP) technique for identifying small mutations and deletions of the dystrophin gene from DNA obtained from blood. These authors studied 93 patients who had a clinical diagnosis of DMD, confirmed on muscle biopsy, but in whom the standard multiplex PCR analysis had failed to recognize mutations. By this novel approach, a mutation was identified in 73 of these 93 patients, therefore improving the ability to detect a mutation in DMD from ~65–70% to ~90% of cases. The drawback of this technique is that each sample has to be run under different temperature conditions, and, considering the large number of exons that comprise the dystrophin gene, even this technique, despite the possibility of its automation, is not very easily applicable on a large scale. By these various methods, it is possible to detect deletions, duplications, and point mutations in the majority of cases and thus help in making diagnoses of both DMD and BMD.

Two novel techniques have replaced the need for Southern blots, multiplex polymerase chain reactions (PCR) or single stranded conformation polymorphism (SSCP) studies, which allows screening of the entire DMD gene for deletions and duplications. The first method uses multiplex amplifiable probe hybridization (MAPH) and analyses all 79 exons of the DMD gene in one assay (DMD MAPH analysis) (White *et al.* 2002). A similar method has been designed, based on multiplex ligation-dependent probe amplification (MLPA) (Schouten *et al.* 2002). The advantage of MLPA, compared to MAPH, is that a lower amount of input DNA is required and MLPA is a one-tube assay. Indeed, this latter technique is now universally used for the detection of deletions and duplications.

None of the methods described above can detect small mutations such as single nucleotide missense, or non-sense changes, or splice site mutations. In order to detect these small mutations, two techniques have been developed.

The first one developed is the protein truncation test (PTT) and is based on an RNA strategy; the others are DNA-based techniques.

The PTT technique involves preparing total RNA from peripheral blood lymphocytes, or better from muscle, which is then translated into protein in an artificial system. The large cDNA of the dystrophin gene is split into several small reactions, and a resultant truncated protein fraction can be easily visualized on electrophoresis. This is nevertheless a time-consuming technique, offered only in very selected cases, in which more recent genomic techniques have failed to identify a causative mutation (see following paragraph).

Direct genomic DNA sequencing of the entire coding region (79 exons) of the dystrophin gene is now available in many regional genetic centres and is the technique of choice in cases with suspected DMD, in whom the MLPA gene test failed to reveal deletions or duplications. This technique readily identifies small mutations, and this, plus the MLPA, can detect most (>98%) of all mutations. Very rare mutations affecting the promoter regions or deep intronic mutations can be studied in research laboratories which have developed microarray-based mutation detection techniques which can also detect rearrangements in the promoter, 3' untranslated region, and introns.

All these genetic tests have their limitations, however, as the possibility of predicting the severity of the phenotype on the basis of the molecular findings is not absolute. The results of genetic testing need therefore to be complemented with the biochemical assay for dystrophin and, of course, the careful assessment of the patient.

Dystrophin studies on muscle biopsy material

The diagnosis of dystrophinopathies, as DMD, BMD, X-linked cardiomyopathy, and manifesting Xp21 carriers are sometimes called, should also be established on the basis of studies of the gene product (dystrophin) in muscle biopsies. This is particularly important in cases in whom the clinical picture is still not fully manifest (for example, very young children). This is possible either by Western blot analysis or immunohistochemistry. The former involves separating proteins from a muscle extract on one-dimensional electrophoresis which is then 'blot-probed' with various monoclonal antibodies to dystrophin. Using MW markers, any difference from normal dystrophin (MW 427 kDa) can be noted and the amount present semi-quantified.

The results of such studies, using various antibodies, reveal a complete absence, or only trace amounts, of dystrophin in DMD. In BMD, on the other hand, dystrophin is present, but in reduced amounts. Furthermore, in the latter disorder, the dystrophin molecule is often of abnormal size, usually with a reduced MW, but occasionally with an increased MW in the case of duplications.

Immunohistochemistry of cryostat sections of muscle provides the most commonly used way of studying muscle dystrophin. In this method, monoclonal antibodies to various parts of the dystrophin molecule (for example, the N-terminal, central rod, and C-terminal regions of the molecule) are raised in an appropriate animal and then labelled with a suitable marker (for example, peroxidase or fluorescent marker). This technique has revealed that, in normal muscle, dystrophin is located close to the sarcolemma, and ultrastructural studies indicate that it forms a lattice-like network adjacent to the membrane. The appearance on cryostat sections is shown in Fig. 4.14. This illustrates that, in

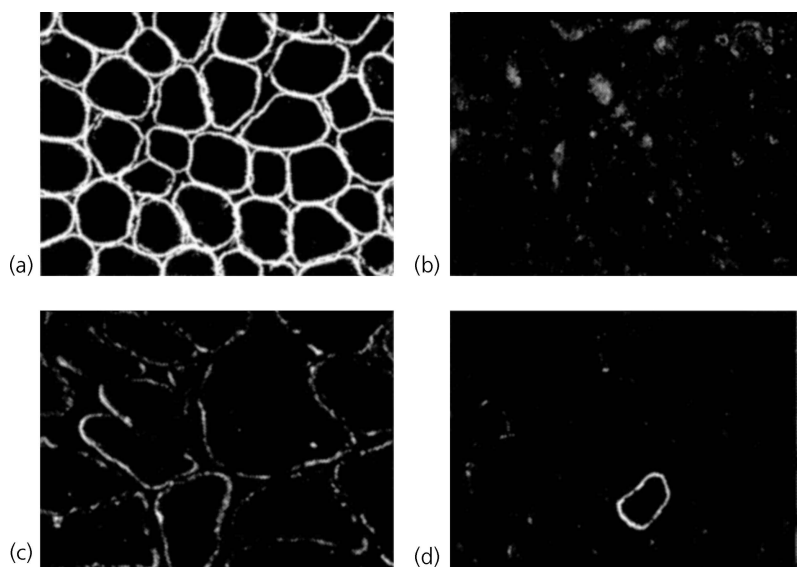


Fig. 4.14 (a) Dystrophin expression in control muscle. (b) Dystrophin in DMD, showing absent expression. (c) Dystrophin in BMD, showing reduced expression. (d) Dystrophin in DMD, showing a revertant fibre.

normal muscle, dystrophin is clearly localized at the periphery of muscle fibres. In DMD, however, the majority of fibres fail to show any staining at all. However, occasional fibres may show some labelling around the periphery. These positive fibres, so-called revertants, usually occur singularly, but may be arranged in clusters, and are found in both familial and non-familial cases. Furthermore, using a panel of antibodies that span the entire dystrophin molecule, in patients with deletions, the positive fibres only stain with antibodies raised to polypeptide sequences outside the deletion, and not those within the deletion. These dystrophin-positive fibres are, most probably, the result of exon skipping that induces a restoration of the reading frame by creating larger in-frame deletions. Other mechanisms, such as second-site in-frame mutations, have also been proposed. In BMD, most fibres do show some staining, but this varies in intensity, both between and within fibres. A list of antibodies commonly used in the diagnosis of DMD is indicated in Fig. 4.15.

A protein similar to dystrophin, and referred to as dystrophin-like protein ('utrophin'), has been identified and is synthesized by a gene on chromosome 6. This protein is normally concentrated in the region of neuromuscular junctions, myotendinous junctions, peripheral nerves, and vasculature of skeletal muscle, but almost absent at the sarcolemma of mature muscle fibres, whilst it is highly expressed in the sarcolemma of fetal and regenerating

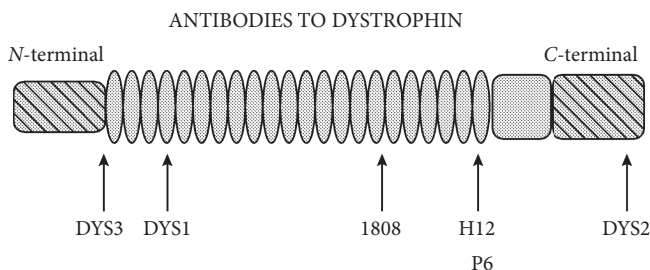


Fig. 4.15 Scheme showing the various monoclonal (DYS3, 1, 2) and polyclonal anti-dystrophin antibodies available.

fibres (see Fig. 4.16). This protein is overexpressed at the sarcolemma in patients with DMD and BMD, and its detection at the sarcolemma in non-regenerating muscle fibres is therefore a useful secondary indicator of a dystrophinopathy (see Fig. 4.16).

In summarizing this section on dystrophin, Western blot analysis and immunohistochemistry on muscle tissue are important techniques for diagnosing a suspected case of Xp21 myopathy. In general, these two techniques complement each other.

From a practical point of view, immunohistochemistry is performed first. It can be performed on a few muscle sections, is rapid, and is reliable if appropriate controls are used. In particular, a marker protein to control for the preservation of the sarcolemma (such as, for example, β -spectrin) should always be used (see Fig. 4.17). Another important point is that multiple antibodies against dystrophin (not just one) have to be used. These have to be directed against different regions of dystrophin, and the most used are those against the N- and C-termini and the rod domain. The use of multiple antibodies increases the

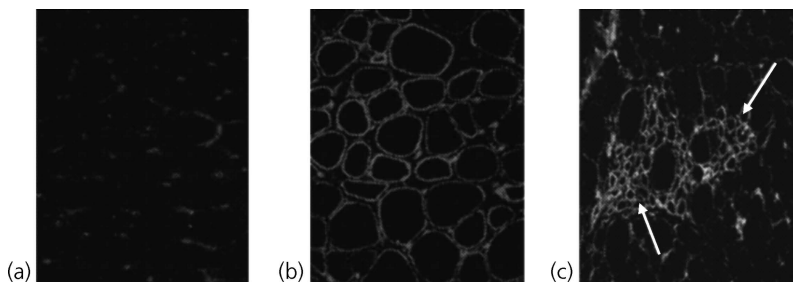


Fig. 4.16 Utrophin. (a) Normal muscle, showing vascular tissue labelled, but not sarcolemma. (b) DMD muscle, with all fibres showing high sarcolemmal labelling. (c) BMD muscle, showing groups of brightly labelled regenerating fibres (arrows), but also labelling on mature, larger fibres.

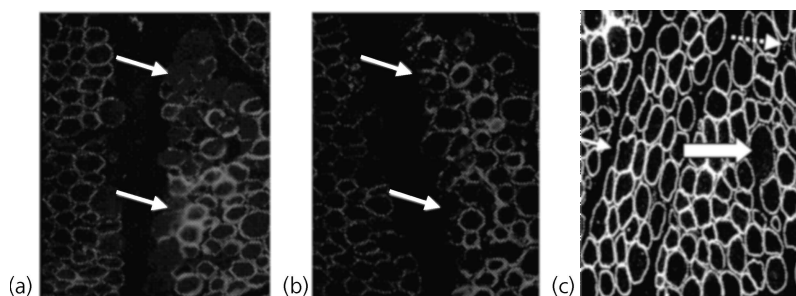


Fig. 4.17 Poor muscle preservation. (a) Dystrophin, with loss of sarcolemmal labelling on some fibres (arrows). (b) Same fibres, also showing reduced β -spectrin due to damage (arrows). (c) DMD β -spectrin. Most fibres with normal sarcolemmal label, occasional necrotic fibre with very little spectrin (large arrow), and occasional regenerating fibre with reduced spectrin (dotted arrow).

sensitivity of the changes observed, but, even more importantly, it helps to differentiate DMD from BMD. In particular, it is important to realize that the presence of an in-frame deletion might give rise to a negative immunohistochemical result if the antibody used is raised against a deleted epitope. Only the immunohistochemical study with a panel of antibodies prevents misdiagnosis in these cases.

If dystrophin is absent or expressed only in trace amounts, there is no need to perform the Western blot analysis. However, if dystrophin is present but appears patchy and not strongly expressed at the periphery of muscle fibres, then a Western blot may help to establish if the patient is affected by BMD, as a secondary abnormality of dystrophin protein expression is a feature of some of the LGMDs.

If the size and abundance of dystrophin on Western blot and its distribution on immunohistochemistry are normal, then this excludes an Xp21 myopathy. A young patient with significant weakness and a muscle biopsy consistent with muscular dystrophy, but with normal dystrophin, is very likely to have an autosomal recessive LGMD of childhood.

The results of these investigations can also provide valuable information of prognostic value. Various studies have concluded that the probability of DMD exceeds 99% if there is a complete absence of dystrophin, whereas the probability of BMD exceeds 95% if dystrophin is present, but of abnormal size (usually smaller) and/or reduced abundance. Patients with <20% of normal levels of dystrophin tend to have an intermediate phenotype, becoming wheelchair-dependent between the ages of 13 and 20 years. Minimal abnormalities of dystrophin expression have also been reported in cases of very mild, late-onset dystrophy, as well as in exceptional healthy individuals with small in-frame

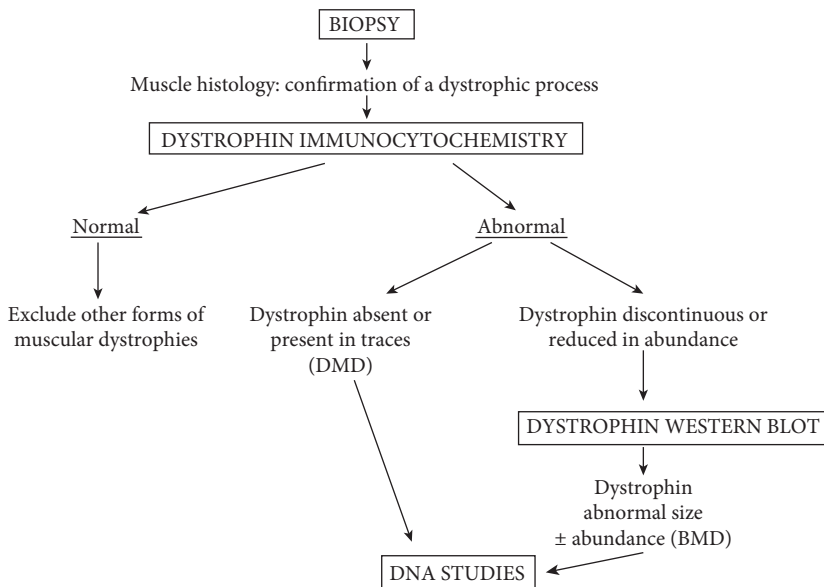


Fig. 4.18 Outline plan for investigating a suspected case of DMD.

deletions of the dystrophin gene. This widens the spectrum of expression of defects at Xp21 and will be discussed further in Chapter 9. An outline plan for investigating a suspected case of Xp21 myopathy is summarized in Fig. 4.18.

Though muscle dystrophin studies can confirm the diagnosis, it is important to establish the precise molecular defect in a case, if reliable prenatal diagnosis is to be offered in a future pregnancy of a female relative of the case.

Other investigations

The diagnosis of DMD can be established in all cases on the basis of the clinical findings, SCK level, and muscle biopsy for histology and dystrophin studies. In the past, a lot of emphasis was placed on electromyography (EMG). However, a confident diagnosis of DMD can now be reached without an EMG. In a child who presents with proximal muscle weakness and markedly raised SCK levels, and in whom the clinical picture does not support the possibility of a diagnosis of dermatomyositis, the most appropriate investigations are a muscle biopsy and genetic testing. An EMG is an unpleasant procedure and does not help to distinguish DMD from other forms of muscular dystrophy.

Muscle ultrasound plays an important role in the investigations of childhood neuromuscular conditions. It has the advantages, compared to other techniques such as computerized tomography (CT) scanning, not commonly used in

children because of the radiation load, or muscle MRI, of not being invasive and of being sufficiently sensitive to demonstrate and localize muscle loss by showing areas of changed density (see Fig. 4.19). These imaging techniques are, however, unlikely to replace more conventional methods of establishing a diagnosis of muscular dystrophy.

As anticipated earlier, muscle MRI is a new and emerging technique to assist diagnosis in many muscle disorders. In DMD and BMD, the clinical and muscle biopsy features are very typical, and muscle MRI is unlikely to be helpful in diagnosing these children. However, in many other muscle conditions, the histopathological findings are non-specific, and, if a specific protein defect is not apparent on histochemical stains, knowing which gene to target for analysis can be difficult. An example might be multi-minicore myopathy where several genetic conditions can give similar clinical and pathological features. Lower limb muscle MRI can be very useful, because specific patterns of skeletal muscle involvement can correlate with the underlying genetic mutation; an example would

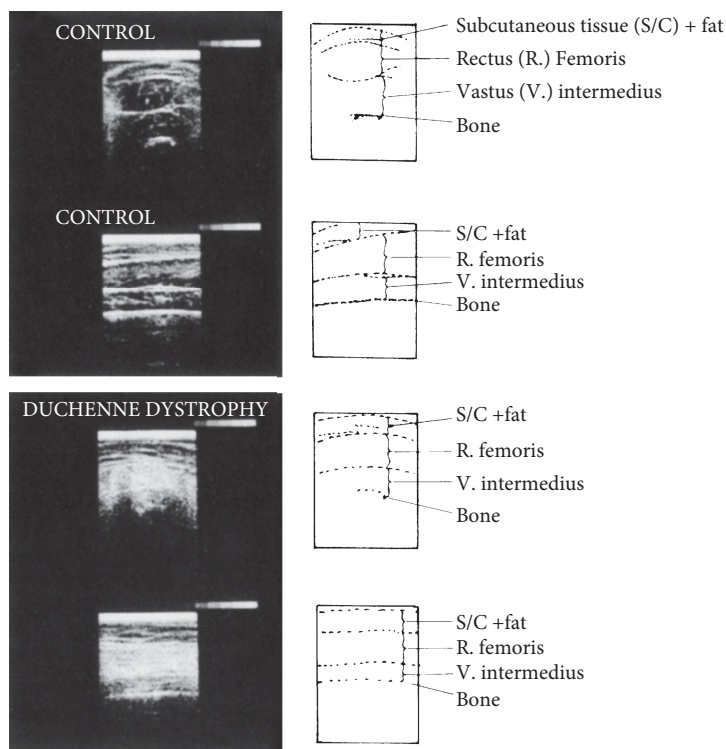


Fig. 4.19 Ultrasonogram of transverse (above) and longitudinal (below) sections of the thigh in a 7-year-old healthy boy and a boy with DMD of the same age. In the latter, there is increased echogenicity throughout all the muscles.

Reproduced courtesy of Dr Adnan Manzur and Professor Victor Dubowitz.

be the ryanodine receptor 1 (RYR-1)-related myopathies (central core myopathy, multi-minicore myopathy, centronuclear myopathy, and malignant hyperthermia susceptibility). T1-weighted axial images through the thigh show prominent involvement of the vasti, compared with the rectus femoris muscle, and of adductor magnus, compared with adductor longus (see Fig. 4.20) (Straub *et al.* 2011).

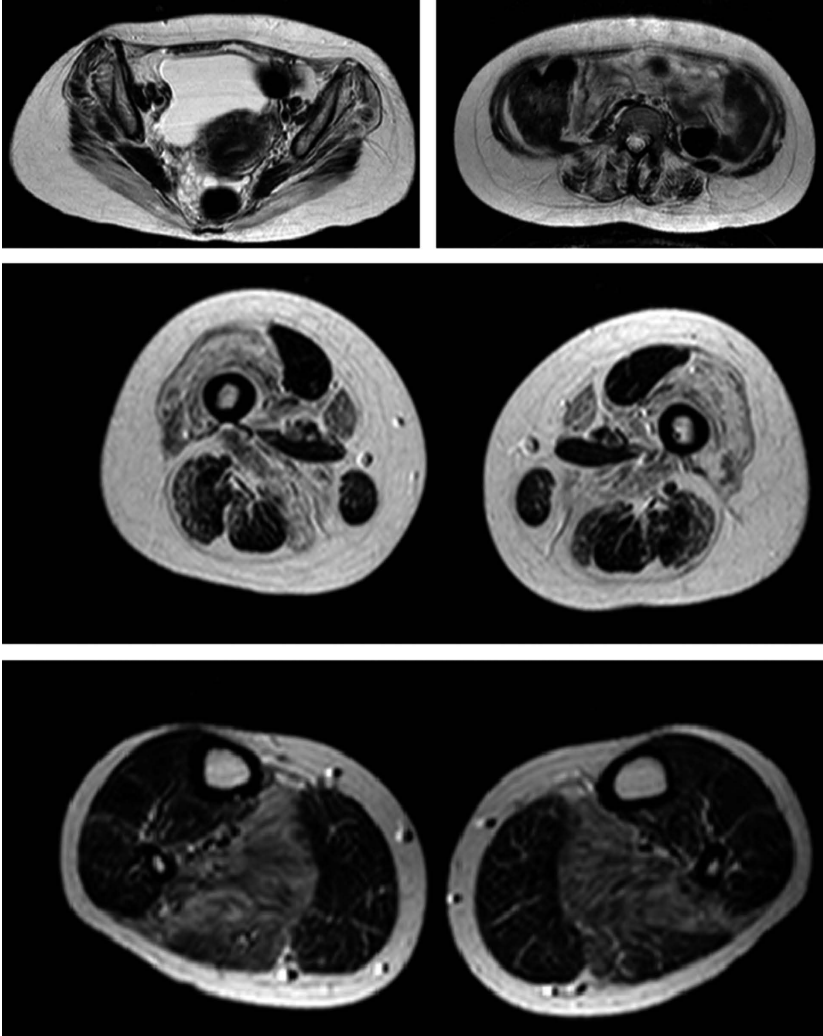


Fig 4.20 Muscle MRI images. Typical pattern of selective muscle involvement detected in a DMD boy using T1 weighted muscle MRI images. In the upper panel (pelvis) there is severe involvement of the glutei; in the intermediate panel (thighs) there is severe involvement of the vasti and off the adductor magnus, while at the calf level (lower panel), there is moderate involvement of the soleus.

Courtesy of Volker Staub.

Summary and conclusions

In the case of an otherwise healthy little boy who presents with evidence of proximal limb-girdle muscle weakness and who has a grossly elevated SCK level (50–100 times normal), the diagnosis of DMD is almost certain. However, there are circumstances where it can be difficult to distinguish DMD from BMD, especially in a very young child. Muscle biopsy, with histochemical stains for dystrophin, will clearly distinguish DMD from BMD.

A significant feature of DMD is the presence of prominent, rounded fibres that stain densely with eosin, the so-called eosinophilic fibres, which contain increased intracellular calcium. The immunostaining for dystrophin has to be performed using a panel of anti-dystrophin antibodies.

Molecular genetic investigations will confirm the mutation in the majority of cases, and this is essential for genetic counselling in the family and for prenatal diagnosis.

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Differential diagnosis

Introduction to differential diagnosis

Disorders that could possibly be confused with DMD include: BMD, several CMDs, LGMD, mild SMA type III, Pompe disease, and dermatomyositis, the last two should be considered in the differential diagnosis of muscular dystrophy, because they are potentially treatable.

The congenital muscular dystrophies

The CMDs are a heterogeneous group of conditions. In all cases, the disorder is evident at birth or within the first 6 months of life, presenting with hypotonia and muscle weakness. The muscle weakness is generalized, although some forms may affect the upper limbs more than the lower limbs, and facial muscles are often affected. Tendon reflexes are usually reduced or absent, and joint contractures may be present at birth or develop later in childhood. Intelligence may be impaired with those CMDs associated with deficient glycosylation of α -dystroglycan (known as ‘dystroglycanopathies’), and there may be structural defects seen on brain MRI such as cobblestone lissencephaly, polymicrogyria, and/or cerebellar cysts. In merosin-deficient CMD, intelligence is normal, but white matter changes are seen on brain MRI. A detailed description of each of the CMDs goes beyond the scope of this book, but further details can be found in the chapter on CMDs in Mercuri and Muntoni (2001, 2012).

The forms that share several presenting features with DMD are: CMD with primary deficiency of the laminin $\alpha 2$ chain of merosin (laminin alpha 2, LAMA2), caused by mutations in the *LAMA2* gene on chromosome 6q22.33 (also known as MDC1A, for muscular dystrophy congenital 1A), especially in children with only partial protein deficiency, as total protein deficiency is typically associated with a much more severe and early-onset variant; and various forms of intermediate severity between CMD and LGMD, due to mutations in genes involved in the glycosylation of dystroglycan (reviewed in Mercuri and Muntoni 2012). The most common genes responsible for these dystroglycanopathy phenotypes, at least in the UK population, are *FKRP* and *ISPD*. The SCK levels are grossly elevated in all these variants, at levels comparable to those found in DMD.

Helpful diagnostic hints for MDC1A (with either total or partial protein deficiency) are the presence of a subclinical demyelinating peripheral neuropathy and of a striking increase in the signal of the white matter on brain MRI. The total absence of LAMA2 is diagnostic of the severe form of MDC1A, whilst a partial protein deficiency is a feature of the milder end of the spectrum of MDC1A. Importantly, a partial LAMA2 deficiency can be found in some of the dystroglycanopathies, but, in addition, in these cases, the study of α -dystroglycan with antibodies that recognize its glycosylation shows marked reduction of the staining. The final diagnosis of these CMD variants requires molecular genetic testing (Mercuri and Muntoni 2012).

Other X-linked muscular dystrophies

Two other X-linked muscular dystrophies are recognized; both are relatively more benign than DMD. The commonest of these is BMD, which occasionally presents in early childhood and can be confused with DMD. Emery–Dreifuss muscular dystrophy presents with more severe joint contractures and slowly progressive muscle weakness and cardiac conduction defects. Danon disease is a very rare X-linked dominant condition; as affected male individuals have elevated CK, proximal weakness, cardiomyopathy, and intellectual disability, they can be also confused for DMD or BMD as well.

Becker-type muscular dystrophy

This condition was first clearly delineated by Becker some 30 years ago (see Chapter 2) (Becker and Kiener 1955) but was recognized to be an allelic variant of DMD, only after the identification of the dystrophin locus.

In contrast to DMD, BMD displays a wide range of clinical expression, ranging from individuals losing the ability to walk in the late teens to individuals who may only experience mild proximal muscle weakness or cramps on exercise and never become significantly physically impaired during the course of their lives. Rare reports of individuals with isolated cardiomyopathy and ‘typical’ BMD deletions are on record, as well as a few families in which the only manifestation of a ‘typical’ BMD deletion was elevated SCK. Toe walking, calf cramps, and even spontaneous myoglobinuria are common presenting symptoms in childhood.

BMD is less common than DMD and has a birth incidence of around 54×10^{-6} in the UK (Bushby *et al.* 1991; Emery 1991). In some populations, however (for example, in Sardinia and Malta; personal observation of one of the authors), the frequency of BMD appears to be higher, approaching that of DMD.

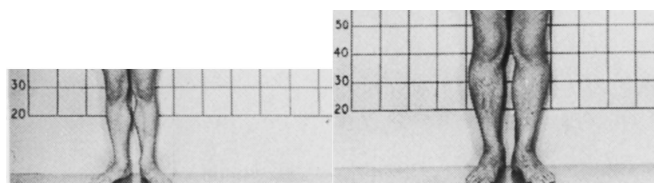


Fig. 5.1 A 6-year-old boy with preclinical BMD (note the enlarged calves) and his affected 26-year-old uncle.

The distribution of muscle wasting and weakness is very similar to that in DMD, and, as in DMD, the hip flexors and quadriceps muscles tend to be affected early in the lower limbs, whilst, in the upper limbs, the serratus, pectoralis, biceps, brachioradialis, and triceps muscles are usually affected first (see Fig. 5.1).

Although weakness almost always begins in the lower limbs, eventually the upper limb musculature becomes affected. Calf enlargement is almost invariably present, and contractures tend to be less severe than those in DMD. Cardiac involvement is a common manifestation, usually not present in the first 15 years of life, but eventually affecting some 60–65% of patients. A small proportion of cases have some impairment of intellect, although a recent report identified frequent behavioural problems and—albeit less frequently—autism (Young *et al.* 2008). The SCK level is substantially raised, especially in the early stages, and gradually falls as the disease progresses. In the preclinical stage of BMD, when the only abnormality is calf enlargement and there is no apparent muscle weakness, the SCK level is grossly elevated to levels comparable to those found in boys with DMD of the same age but who are clinically affected.

The very high SCK levels early in the course of BMD and the overlap in the age of onset with DMD can present a diagnostic dilemma in isolated cases. Points of clinical value in distinguishing between the two disorders have been mentioned in Chapter 3 and essentially can be summarized as follows.

- ◆ Children with DMD are never able to run properly, hop on one leg, lift their head off the bed, or get up from a sitting position on the floor if asked to keep their arms folded.
- ◆ Conversely, children affected with BMD are typically able to run, hop, lift the head off the bed, and get up from the floor without the Gowers' manoeuvre, at least in the early phases of the disease.

Later, the two disorders differ in the age of becoming confined to a wheelchair; in DMD, this usually occurs by age 13, but, in BMD, after age 16, with a reported mean age of loss of independent ambulation around 37 years (Bushby and Gardner-Medwin 1993).

Apparent Duchenne muscular dystrophy and Becker muscular dystrophy in the same family

The occurrence of patients with classical DMD and others with BMD within the same family is very rare. In at least one instance, a *de novo* independent additional mutation in the dystrophin gene has been reported in a child with DMD in a BMD family, providing the molecular basis for the discrepant phenotype. Another more common mechanism occurs in siblings with BMD with out-of-frame deletions where a discordant phenotype is the different occurrence of revertant fibres (individual fibres that express dystrophin) generated by exon skipping (see Chapter 13 for a more detailed explanation on exon skipping). Exon skipping can induce a larger in-frame functional deletion, partially restoring dystrophin function (see Chapter 13).

The diagnostic approach to differentiating DMD from BMD on clinical and biochemical grounds was discussed in Chapter 3.

X-linked muscular dystrophy with early contractures and cardiomyopathy (Emery–Dreifuss type)

In 1961, Dreifuss and Hogan described a large family in Virginia in the United States (US) with an X-linked form of muscular dystrophy, which they considered at the time to be a benign type of DMD. However, on reinvestigating the family a few years later, this seemed, to one of us, to be a very different disease from either DMD or BMD, and a report setting out the differences was published in 1966 (Emery and Dreifuss 1966). The onset is in early childhood and is marked by progressive muscle wasting and weakness that, in the beginning, affects the lower limbs more than the upper limbs. The progression is relatively slow, and most affected individuals survive into middle age, with varying degrees of incapacity (Emery 1989). There does not appear to be any intellectual impairment. The SCK level is usually slightly raised but, even in the early stages, never approaches the grossly elevated levels found in DMD. The distinctive features of this disorder are as follows.

- ◆ Early contractures of the elbows and Achilles tendons, and later the posterior cervical muscles.
- ◆ Muscle weakness is more proximal (scapulohumeral) in the upper limbs, and distal (anterior tibial and peroneal muscles) in the lower limbs, at least in the beginning.
- ◆ There is no calf pseudohypertrophy.
- ◆ Myocardial involvement with cardiac conduction defects is a frequent and important feature (see Fig. 5.2). Provided the diagnosis is made sufficiently early, the insertion of a cardiac pacemaker can be lifesaving.

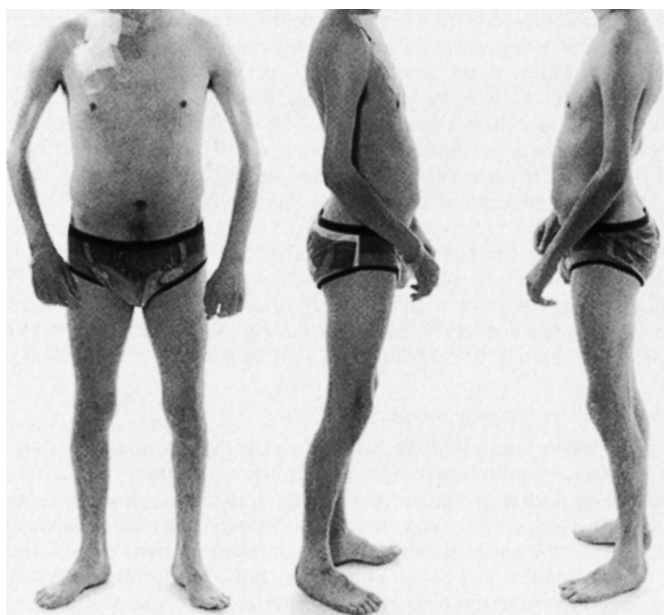


Fig. 5.2 A 17-year-old boy with X-linked muscular dystrophy with early contractures and cardiomyopathy. Note the flexion contractures of the elbows and wasting of the lower legs. A cardiac pacemaker has been inserted.

The gene responsible for X-linked Emery–Dreifuss muscular dystrophy is the *STA* gene, located in Xq28, which encodes for emerin, a nuclear envelope protein. The diagnosis of X-linked Emery–Dreifuss muscular dystrophy is based on the expression of emerin, using immunocytochemistry, in muscle or skin samples—in affected males, typically there is no expression. The diagnosis should be confirmed by appropriate molecular genetic studies. Recently, the gene for an autosomal dominant phenocopy of the X-linked Emery–Dreifuss muscular dystrophy variant has been localized to chromosome 1q where the *LMNA* gene encodes another nuclear envelope protein lamin A/C. Mutations are usually dominant missense mutations, and the expression of the normal allele makes interpretation of the immunohistochemistry or Western blot difficult. The diagnosis is therefore dependent on the identification of mutations in the *LMNA* gene. A rare severe autosomal recessive form, due to mutations of the *LMNA* gene, also exists. Mutations in this gene unexpectedly also result in isolated dilated cardiomyopathy with conduction system disease, or in partial lipodystrophy, or in autosomal recessive axonal neuropathy. Mutations in yet another X-linked gene *FHL1* can also result in an Emery–Dreifuss phenotype.

Autosomal recessive limb-girdle muscular dystrophy of childhood

Several autosomal recessive LGMDs are clinically almost indistinguishable from DMD. They are mostly due to mutations in the sarcoglycan genes (α , β , γ , and δ , responsible for LGMD2D, LGMD2E, LGMD2C, and LGMD2F, respectively). These genes encode four sarcolemmal proteins that are part of the dystrophin complex. It is therefore not entirely surprising that the phenotype of children with these disorders, also referred to as severe childhood autosomal recessive muscular dystrophy (SCARMD), is so similar to that of children with DMD.

There are, however, subtle clinical differences that can help to distinguish DMD from SCARMD on clinical grounds. Early motor milestones are typically delayed in DMD, whilst they are normal in children with SCARMD. Whilst, in DMD, children are never able to run or jump, these functional abilities are usually preserved at the beginning in most patients with SCARMD. Intelligence is always normal in SCARMD, and height and head circumference are within the normal ranges. SCK levels are similar to those found in DMD.

The distribution of weakness is very similar, but there are some differences. In SCARMD, there is typically more significant scapular winging and weakness, compared to DMD, and more substantial weakness of the hip and trunk extensors with an increased lordotic posture, compared to that generally observed in children with DMD. The peroneal and tibialis anterior muscles are also more affected, compared to those of children with DMD, and it is not unusual to observe foot drop in the ambulant phases of children with SCARMD. The quadriceps muscle is more affected than the hamstrings in DMD, but the reverse is generally found in SCARMD.

Cardiac function is usually better preserved in SCARMD than in DMD, with the possible exception of LGMD2F, in which early and severe cardiomyopathy has been reported. This is of interest also, considering that the δ -sarcoglycan gene is responsible for LGMD2F, but also for the cardiomyopathic hamster, an animal model of dilated cardiomyopathy. Respiratory function is also better preserved in SCARMD, compared to DMD. The main similarities and differences between DMD and SCARMD are summarized in Table 5.1.

The diagnosis ultimately depends on immunocytochemical and Western blot analysis of the sarcoglycan proteins, followed by molecular genetic testing of the relevant genes. It is important to be aware that dystrophin expression on immunocytochemistry can be secondarily depressed in patients with mutations in the β - and δ -sarcoglycan genes, probably because the lack of these two proteins causes a more significant destabilization of the complex.

Table 5.1 Main clinical features of DMD and SCARMD

Clinical features	DMD	SCARMD
Calf hypertrophy	Common	Common
Weak foot dorsiflexion	Rare	Common
Lumbar lordosis	Moderate	Severe
Scapular winging	Mild	Moderate to severe
Intellect	Normal to mild mental retardation	Normal
SCK levels	>10 times normal	>10 times normal
Respiratory failure	Late teens	Late twenties

Other forms of autosomal recessive muscular dystrophy, with clinical features very similar to those of DMD, are those related to genes involved in dystroglycanopathies. LGMD2I, due to mutations in the fukutin-related protein gene (*FKRP*), is the most common form in the UK, followed by the variant secondary to mutations in *ISPD*. Affected patients may present in childhood with proximal weakness, calf hypertrophy, and marked elevation of SCK. Cardiac involvement, in the form of dilated cardiomyopathy, is also frequently observed; thus, the differential diagnosis is DMD and BMD. As is the case with BMD, muscle cramps and myoglobinuria may be the presenting features of LGMD2I.

A full discussion of the autosomal recessive LGMDs is found in Bushby (2001).

The manifesting carrier of Duchenne muscular dystrophy

Female carriers of DMD may occasionally manifest certain features of the disease, and there is not always a history of muscular dystrophy in the family. In 1879, Gowers reported an affected girl in a clearly X-linked pedigree (Case 8), the sister of an affected boy (Case 15), and an isolated case (Case 30). Since then, there have been many similar reports, with manifestations of the disease ranging from calf enlargement, through varying degrees of muscle weakness, to occasionally severe incapacity.

It has been estimated that between 5 and 10% of DMD carriers have some degree of weakness. But this may be slight and only evident on careful clinical examination. Calf enlargement has often been emphasized but is, in fact, an unreliable sign, and, when present, it is usually asymmetric. Actual measurements of the calf size may reveal no significant difference between controls

and definite carriers. An early feature may be muscle cramps, again often asymmetric, which can be confused with a metabolic or inflammatory myopathy, especially when there is no family history of DMD. The onset of weakness varies considerably and may develop in childhood or may not become evident until adult life, and the weakness may be progressive or remain static. In many ways, the distribution of weakness resembles that seen in adult LGMD but differs in that pseudohypertrophy is usually present. The weakness is often asymmetric; electrocardiographic abnormalities similar to those seen in affected boys can occur, and the SCK level is invariably raised and occasionally may even approach levels found in affected boys. On the other hand, some female carriers may have very high SCK levels yet have no muscle weakness at all. Most importantly, the heart may also be affected, and a proportion may develop dilated cardiomyopathy, even in the absence of any overt weakness (Grain *et al.* 2001). For this reason, regular echocardiography in adult female carriers of DMD is recommended.

It is important to distinguish a manifesting adult female carrier from a woman with autosomal recessive LGMD (see Table 5.2), both of which occur with very roughly the same frequency, because genetic counselling in these two situations will be quite different. The risks to the sons of a manifesting carrier of X-linked DMD will be 50%, but the risks to the offspring of a woman affected with autosomal recessive LGMD will be negligible. Manifesting carriers of BMD are exceptional.

Manifesting carriers of DMD have occasionally been described in the same family, and this has also been observed in other X-linked disorders such as Fabry's disease. DMD extending over seven generations has been described in a large Swiss family with 14 affected males and no fewer than

Table 5.2 Differentiation between a manifesting carrier of DMD and a woman with LGMD

Symptom or sign	DMD carrier	LGMD
Pseudohypertrophy	>80%	Rare*
Muscle weakness	Often asymmetric	Rarely asymmetric
ECG abnormalities (R-S in V ₁ increased)	5–10%	Usually normal
Dilated cardiomyopathy	5–8%	Usually normal*
SCK level elevated	>95% (often very high)	<50% (rarely high)
Muscle dystrophin	Mosaicism	Normal
Dystrophin DNA analysis	Abnormal	Normal

* With the exception of LGMD21.

four manifesting carriers. Since such manifestations are a consequence of random X-inactivation, their familial occurrence suggests that this process may be under genetic control. In fact, in the mouse, there is an X-linked locus that controls X-inactivation (X chromosome-controlling element, Xce).

Such manifestations in heterozygous females can be explained in terms of random inactivation of the X chromosome. In those women with no clinical manifestations and a low SCK level, the active X chromosome in most cells is the one bearing the normal gene, whereas, in those women with manifestations of the disease and a high SCK level, the active X chromosome in most cells is the one bearing the muscular dystrophy gene.

Manifesting carriers of DMD have a so-called mosaic expression of dystrophin in muscle. Such mosaicism, however, is rarely found in female carriers without symptoms. Mosaicism of dystrophin expression is believed to result from the formation of multinucleate muscle fibres from the fusion of uninucleate myoblasts, in some of which the normal X chromosome is active (and therefore dystrophin-positive), whilst, in others, the abnormal X chromosome is active (and therefore dystrophin-negative). However, though some authors have hypothesized the existence of two distinct populations of muscle fibres in carriers, one normal and the other abnormal, a wide spectrum of abnormalities can be seen instead. These range from muscle fibres that appear to be normal to those that are clearly undergoing necrosis. The explanation lies in the way multinucleate muscle fibres originate and develop. If fetal myotomes were mosaics of nuclei, in some of which the active X chromosome is the one bearing the normal gene and, in others, the active X chromosome is the one bearing the Duchenne gene, then fusion between mononucleate myoblasts derived from the myotomes would result in muscle fibres possessing different proportions of the two types of nuclei. The proportion in any one fibre would be determined, to some extent, by the proportion in the original myotome. The proportion of nuclei in any muscle fibre, in which the active X chromosome is the one bearing the Duchenne gene, presumably determines the degree of abnormality in that fibre (see Fig. 5.3).

This interpretation has been recently confirmed by detailed immunohistochemical studies on dystrophin expression. Abnormal dystrophin expression can be clearly identified in manifesting carriers, who often show a population of entirely dystrophin-negative fibres, of dystrophin-positive fibres, and of a third mixed population with variable levels of expression of dystrophin (see Chapter 11). Whilst there is, broadly speaking, a correlation between the degree of X-inactivation, and therefore the degree of skewed expression of dystrophin in a mosaic pattern, and clinical severity, it is important to acknowledge that this is not per se the only determinant of clinical severity; hence, care should be

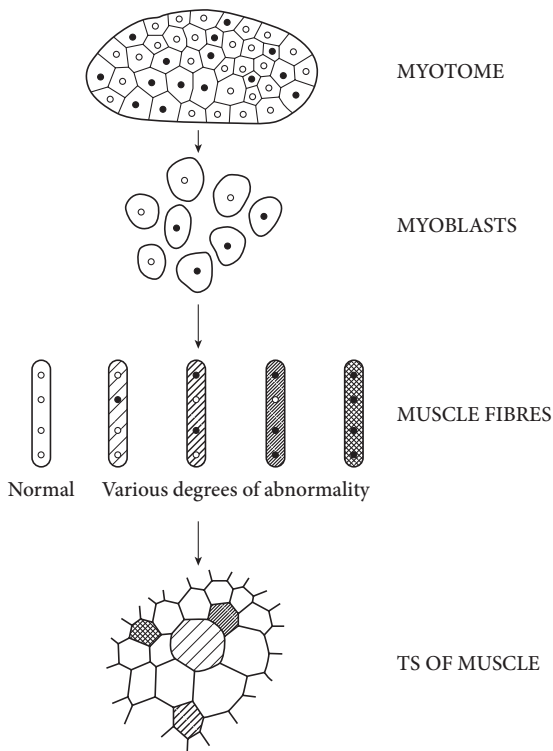


Fig. 5.3 Possible explanation for the muscle histological findings in carriers of DMD. (Open circles) Nuclei in which the active X chromosome bears the normal gene. (Black circles) Nuclei in which the active X chromosome bears the Duchenne gene. TS, transverse section.

exerted when prognosticating the clinical severity in manifesting DMD carriers (Brioschi *et al.* 2012; Mercier *et al.* 2013).

But, in non-manifesting carriers, variability in immunostaining is infrequent, and dystrophin-negative fibres are rare, especially if the SCK level is normal. Interestingly, utrophin is also often upregulated in muscle fibres of carriers, despite the apparent normal level of dystrophin, and therefore represents an additional marker that should be studied.

It is important to emphasize that secondary abnormalities of dystrophin immunostaining have been noticed in several cases of patients with sarcoglycanopathy and, in particular, in cases with mutations in the β - and δ -sarcoglycan genes, but also some of the dystroglycanopathy cases. In a symptomatic female patient without a clear mosaic pattern of dystrophin expression, therefore one of the recessive sarcoglycanopathies cannot be excluded. Further helpful hints come from the upregulation of utrophin, which is usual in cases of Xp21 muscular dystrophies, but not in sarcoglycanopathies (see Fig. 5.4).

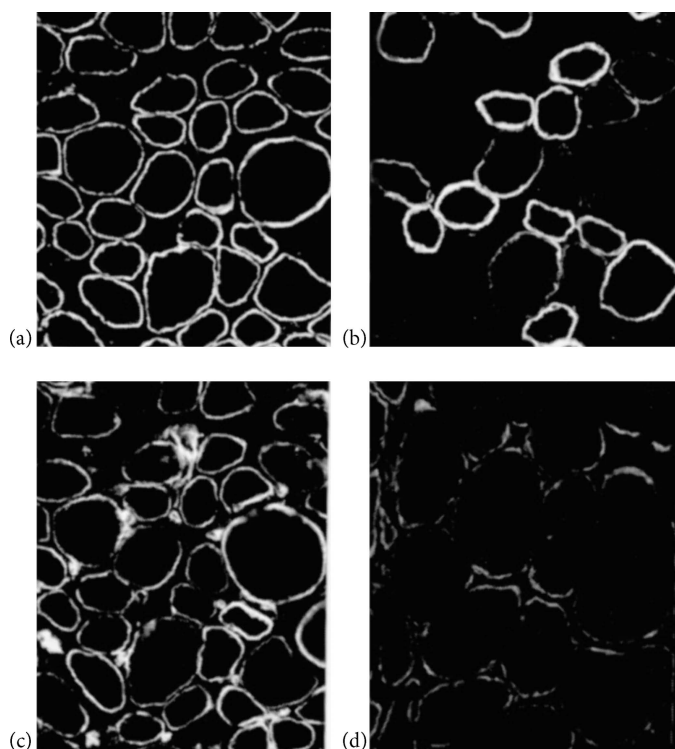


Fig. 5.4 (a)–(c) Duchenne carrier. (d) β -sarcoglycanopathy. (a) Regular β -spectrin expression, indicating good sarcolemmal preservation. (b) Dystrophin-positive and dystrophin-negative fibres in a DMD carrier. (c) Utrophin expression in all fibres in a DMD carrier. (d) Secondary reduction of dystrophin in a patient with β -sarcoglycanopathy.

Heterozygous identical twins

Over the years, a number of female identical (monozygotic, MZ) twins have been described who are discordant for manifestations for various X-linked recessive disorders, most notably DMD, but also colour blindness, haemophilia B, glucose-6-phosphate dehydrogenase deficiency, and Hunter's disease. The simplest explanation is that, during the process of twinning, by chance, the affected twin received a greater proportion of cells in which the active X chromosome was the one bearing the mutant gene. Certainly, there is experimental evidence that, in these cases, the clinical expression of the disease in the affected twin is the consequence of the inactivation of the normal X chromosome in most of her cells, as compared with those of her unaffected twin sister. But why is the expression limited to only one twin of a pair, and why is the concordant expression in MZ heterozygous female twins

extremely uncommon? This may be because the twinning event occurs after X-inactivation, and, in these cases, in some way, non-random X-inactivation itself causes the twinning process. But discordance in female MZ twins has also been reported in certain autosomal disorders. The situation is therefore not clear. Lubinsky and Hall (1991) suggested that genomic imprinting, monozygous twinning, and X-inactivation may all be somehow related and ‘... their interactions may provide important clues to the nature of early developmental processes.’

Danon disease

Danon disease is an X-linked dominant condition due to mutations in *LAMP2* gene, located on the long arm of chromosome X (Xq24). Affected males present in childhood with proximal muscle weakness, the arms often more affected than the legs, elevated SCK (4–35 times), and dilated, or more often restricted, cardiomyopathy. Intellectual disability is very common, but not always present, in affected males. Carrier females also develop features of the condition, although typically at an older age than affected males in the same family.

The spinal muscular atrophies

The SMAs are defined as a group of inherited diseases, in which there is degeneration of the anterior horn cells (lower motor neurons) of the spinal cord, and often the bulbar motor nuclei, but with no evidence of pyramidal tract or peripheral nerve involvement. This group of diseases excludes motor neuron disease (progressive bulbar palsy, progressive muscular atrophy, and amyotrophic lateral sclerosis) and its variants, as well as the peripheral neuropathies. The SMAs are a heterogeneous group of disorders and vary considerably in their clinical presentation and mode of inheritance. Distal and proximal variants are recognized, but the most common form is the chromosome 5q-linked form of proximal SMA due to mutations in the *SMN* gene. This can be subdivided into several variants, depending on the disease severity. In type I (also known as Werdnig–Hoffmann disease), affected children present at birth or in the first 6 months and are never able to sit independently. In type II, children present before the eighteenth month of life and can sit but cannot walk. In type III (also known as Kugelberg–Wielander disease), in which the onset is usually after the eighteenth month of life, ambulation is possible but may be lost later. Some authors also recognize a fourth type, with the onset after the age of 30 years (SMA IV). These four subtypes are allelic, that is, due to mutations in the same gene—the *SMN1* gene. Table 5.3 summarizes the main features of these common forms of SMA.

Table 5.3 Distinguishing features of the different forms of proximal SMA

Type	Age (usual) of onset	Survival	Ability to sit without support	Muscle fasciculations	SCK
I (infantile)	<6 months	<2 years	Never	+/-	Normal
II (intermediate)	<18 months	Variable*	Yes	+/-	Usually normal
III (juvenile)	>18 months	Adulthood	Yes	++	Often raised
IV (adult)	>30 years	Normal	Yes	++	Often raised

* Survival into adolescence and adulthood increasingly possible.

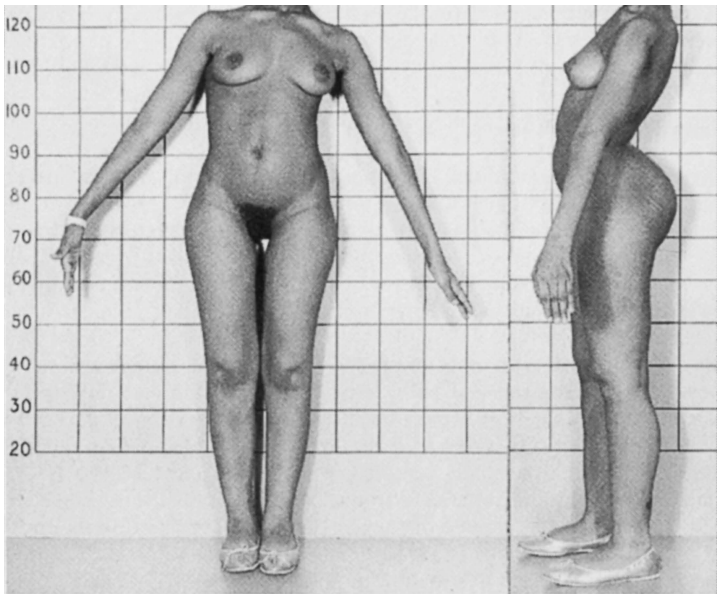


Fig. 5.5 A 17-year-old girl with SMA type III (Kugelberg–Welander disease). Note the marked spinal lumbar lordosis and wasting of the left deltoid muscle. An older and two younger brothers were also affected.

From a practical point of view, the only form that might possibly be confused with DMD, or more usually BMD, is SMA type III (see Fig. 5.5). The clinical presentation may closely resemble that of DMD/BMD; weakness first affects the pelvic girdle musculature, and patients often present with a tendency to fall and a waddling gait. Later, the pectoral girdle, neck, trunk, and distal limb muscles also become affected. Interestingly, muscle weakness, at least in the early stages, is often asymmetric, which is unlike muscular dystrophy; pseudohypertrophy of the calf muscles is uncommon but can occur, whilst muscle fasciculations, which are often present, are a useful diagnostic sign. A fine

tremor of the outstretched hands is common, and minipolymyoclonus, intermittent and irregular movement that is sufficient to produce visible movement of the joints and head, may also be present. The severity of the disease is very variable, even within families, although many become wheelchair-dependent during the adolescent growth spurt. The SCK level is very rarely grossly elevated, but it can be moderately elevated. In most cases, EMG confirms the neurogenic nature of the disorder. A useful sign is the presence of trembling of the electrocardiogram (ECG) baseline, due to fasciculation of intercostal muscles. The diagnosis of SMA type III can now be rapidly and confidently established by DNA analysis, as >90% of affected individuals have a mutation affecting the telomeric version of the *SMN1* gene.

Summary and conclusions

Several disorders can mimic DMD, the most usual of which is BMD presenting in early years. However, an important point of distinction is that, in BMD, the abilities to lift the head off the bed, to run and hop, and to get up from the floor, without the classical difficulties observed in DMD, are preserved in the early phases of the disorder. Furthermore, muscle dystrophin is virtually absent in DMD, whereas, in BMD, dystrophin is present but abnormal.

Other neuromuscular conditions presenting in early childhood include CMDs and the mild form of SMA (SMA III). Among the muscular dystrophies, the congenital forms are unlikely to lead to confusion, because, in general, they are evident at birth or in the neonatal period with severe hypotonia and generalized muscle weakness. However, milder allelic variants of some of these do overlap, from a clinical point of view, with both DMD and LGMDs. The best example is given by mutations in the *FKRP* gene, located on chromosome 19, responsible both for a form of CMD (MDC1C) and a form of LGMD (LGMD2I) that presents with proximal muscle weakness, calf and thigh hypertrophy, grossly elevated SCK, and frequently a dilated cardiomyopathy. Among the other X-linked muscular dystrophies, the benign form, associated with early contractures and conduction system disease (Emery–Dreifuss muscular dystrophy), is so distinctive clinically that this too is unlikely to cause confusion but because of the very high SCK levels early in the course of the disease, overlap in age at onset, and similar pattern of muscle involvement.

A particularly difficult problem is posed by SCARMD when only a boy is affected in the family. One can suspect a severe autosomal recessive LGMD in a child with normal early motor milestones and intelligence, significant scapular

winging and weakness, and significant hip extensor and peroneal muscle weakness. The incidence of severe autosomal recessive LGMD is, however, extremely low, compared to DMD, in most ethnic groups, and muscle immunohistochemical studies will confirm which protein is primarily involved, suggesting the final diagnosis that will eventually be confirmed by the appropriate molecular genetic investigations.

Finally, the only form of SMA that might possibly be confused with DMD is the proximal juvenile form (type III, Kugelberg–Wielander disease). Points that would suggest this disease, rather than DMD, would be some asymmetry of muscle involvement, the absence of pseudohypertrophy, and evidence of muscle fasciculations. SCK levels are significantly (5–10 times normal) elevated in rare cases but more frequently normal. In most cases, EMG and muscle histology will confirm the neurogenic nature of the disorder. And again, molecular genetic testing will confirm the diagnosis.

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Chapter 6

Involvement of tissues other than skeletal muscle

Introduction to involvement of tissues other than skeletal muscle

The muscular dystrophies have often been described as primary diseases of muscle. The term 'primary', in this context, could have two interpretations, either that muscle is the most obviously affected tissue, which is certainly true, or that the fundamental molecular defect is expressed only in skeletal muscle, which is patently not true. In recent years, it has been shown that significant abnormalities can be found in a variety of tissues, quite apart from skeletal muscle. This is perhaps not unexpected, since the abnormal gene is transcribed in a variety of cells of the body.

The variety of manifestations of a genetic disease can result from the pleiotropic effects of a single gene or at the molecular level, either from the involvement of adjacent genes that control other phenotypic features or from different point mutations within the same gene.

Pleiotropy refers to the multiple effects that a gene mutation may have as a trail of consequences leading on from the basic defect. An excellent example of this is provided by sickle-cell anaemia, wherein the responsible gene mutation results in sickle-cell haemoglobin, which is less soluble than normal haemoglobin and therefore tends to crystallize out, resulting in deformation of the red cell which becomes sickle-shaped. These abnormal cells are then destroyed (haemolysed), resulting in anaemia. But, at the same time, they also tend to clump together, thereby obstructing small arteries, resulting in ischaemia of tissues with a variety of consequences, including attacks of abdominal pain, splenic infarction, limb pains, osteomyelitis, cerebrovascular accidents, haematuria, renal failure, 'pneumonic' episodes, and heart failure. Whilst most of the features of DMD can be accounted for by the defect of dystrophin in the target tissue (that is, the absence of dystrophin in skeletal muscle is related to the development of the muscle damage; the absence of dystrophin in the cardiac muscle is related to cardiomyopathy; the abnormal expression of dystrophin in the brain is clearly involved in the mental retardation observed in

affected children), some manifestations of the disease are not so easily explained. For example, the development of muscle degeneration is not simply the result of dystrophin deficiency, as some muscles are spared. The absence of dystrophin, in fact, triggers a **complex cascade of events** that includes muscle degeneration.

An association of different genetic disorders in the same individual can occur purely by chance such as the reported occurrence in DMD of haemophilia, trisomy 21, or facioscapulohumeral muscular dystrophy. However, very occasionally, such an association may result from the deletion of genetic material, involving adjacent genes that are responsible for different diseases (contiguous gene syndrome or overlapping phenotypes). Several associations of DMD with disorders due to genes closely located on Xp21 have been described. These include a boy with DMD with chronic granulomatous disease and retinitis pigmentosa, and another with DMD, glycerol kinase deficiency, and adrenal insufficiency. Often, cytogenetic studies reveal that these children have a small, but visible, interstitial deletion of the short arm of the X chromosome (Xp21) that involved the loss of genetic material from all three of the adjacent genes responsible for these different diseases (see Chapter 9). However, this sort of molecular abnormality is rare and very much the exception. A significant part of the variability observed in clinical practice among different children with DMD is due to differences in the site and extent of mutations within the dystrophin gene at Xp21.

Whatever the mechanism, it is important in patient management to appreciate that DMD is a multisystem disease and that a variety of tissues, other than skeletal muscle, can be affected, including cardiac muscle, smooth muscle, the brain, and possibly the retina. It is also important in genetic counselling to acquaint 'would-be parents' with the possible consequences of the disease in an affected child so that they can better appreciate the full extent of the problem.

Smooth muscle

Smooth muscle is affected in DMD; paralytic ileus, volvulus, and gastric dilatation have all been reported and are often terminal events. In addition, severe constipation is a frequent complication in adults with DMD and can be complicated with anal fissure. Occasional occurrence of type 2 diabetes and bladder paralysis has also been reported, and, more recently, with prolonged survival of some DMD adults into the fourth decade, renal failure may be a cause of death (Matsumura *et al.* 2011). Detailed autopsy studies have shown that the smooth muscle of the gastrointestinal tract often shows variation in fibre size, atrophy and loss of muscle fibres, and areas of fibrosis. These changes are comparable, in

many ways, to those seen in the affected skeletal muscle and have occasionally been found in cases who did not have any relevant gastrointestinal symptoms in life. From a functional point of view, however, children with DMD are known to suffer much more severe blood loss following major surgery, such as spinal surgery, compared to that experienced by other disease controls. In the absence of a clotting problem in these patients, it has been hypothesized (but not proved) that this abnormality is the result of the difficulty in arterial and arteriolar vasoconstriction of children with DMD. Animal studies have confirmed the presence of dystrophin in endothelial smooth muscle (Loufrani *et al.* 2011). A significant deficiency of platelet adhesion and ristocetin-induced aggregation has also been reported, and this might contribute to the blood loss following major surgery (Forst *et al.* 1998).

Cardiac muscle

Cardiac involvement is an important cause of mortality in DMD. From an early age (already at presentation), there is very often a persistent sinus tachycardia. Clinically apparent cardiomyopathy usually first becomes evident with the loss of independent ambulation and increases in incidence with age thereafter, although there are reports of lethal cardiomyopathy occurring in much younger children. Mitral valve prolapse has been recorded in up to a quarter of affected boys, auscultatory evidence of which can be confirmed by echocardiography. Whilst it is estimated that almost all DMD patients have signs of cardiac involvement in the late stages of their disease, progressive heart failure is rare (~15% of cases). Plasma atrial natriuretic peptide levels have been suggested as a possible means of evaluating cardiac function in the disease, as in other cardiomyopathies. However, it is not a useful screening test for cardiomyopathy in DMD, as routine screening of affected boys showed no abnormality (Ramaciotti *et al.* 2002).

At autopsy, microscopic studies of cardiac muscle reveal features that resemble those seen in skeletal muscle, with variation in the fibre size, fragmentation of muscle fibres, replacement by connective tissue, and some fatty infiltration. Such changes have even been found in patients who did not necessarily have any symptoms of heart disease during life. Fibrosis appears to be a particularly important feature (see Fig. 6.1). It begins in the outer myocardium, involving the more posterobasal part of the outer free wall of the left ventricle. At first, fibrosis appears in discrete small areas but eventually becomes more diffuse and involves most of the outer half of the ventricular wall. The right ventricle and atria are rarely reported to be involved, although careful monitoring identifies both in the mouse model of DMD and, in boys, the involvement of the right

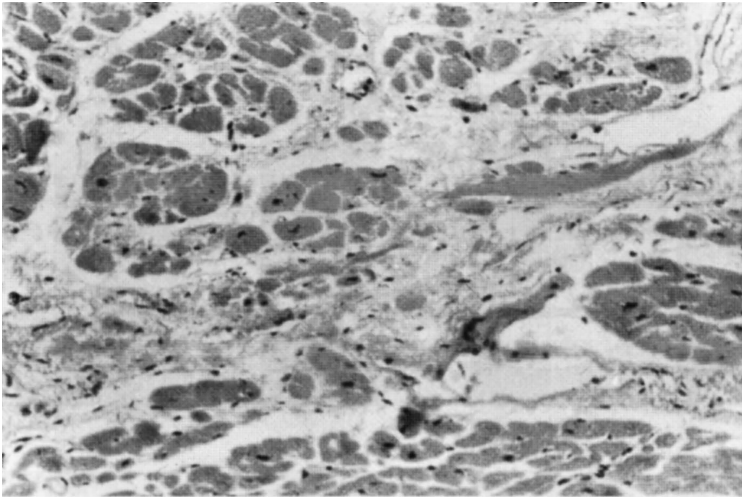


Fig. 6.1 Histological appearance of cardiac muscle from the left ventricle from a 21-year-old boy with DMD. Note the variation in fibre size and the marked increase in connective tissue (haematoxylin and eosin).

ventricle as well (Bosser *et al.* 2004). Recently, at Great Ormond Street Hospital, routine cardiac MRI in DMD children, as part of preoperative screening for scoliosis surgery, has confirmed the frequent involvement of the right ventricle (Dr Matthew Fenton, unpublished data). A similar pattern of myocardial fibrosis is also seen in BMD and X-linked dilated cardiomyopathy (XLDCM).

Early in the course of the disease, cardiac catheterization studies have shown few consistent abnormalities, but, in severely affected boys approaching cardiac failure, significantly elevated right atrial and right ventricular end-diastolic pressures have been recorded. Non-invasive techniques, such as ballistocardiography, vectorcardiography, echocardiography, and, more recently, MRI and tissue Doppler studies, have all documented variable degrees of cardiac involvement. With this latter technique, significant abnormalities can already be demonstrated in the early ambulant phases of the disease. ECG has been used extensively over the years and therefore merits some special consideration. The first description of ECG abnormalities in muscular dystrophy dates back to 1929. Since then, a variety of ECG changes have been observed and are listed in Box 6.1. Evidence of defective cardiac conduction has also been reported, and its significance increases with age, typically representing a concern only in the young men.

Numerous attempts have been made to determine if any particular ECG pattern is specific to DMD. Ishikawa and his colleagues in Japan (1982) have suggested that high-frequency ‘notches’ on the QRS complexes can be used for

Box 6.1 ECG abnormalities arranged in decreasing order of frequency in DMD

Tall R waves in V_1 :

- ◆ R/S ratio \uparrow ;
- ◆ [R-S] \uparrow .

Shortened P-R interval

Deep Q waves in V_{5-6}

Complex RSr^1 , right bundle branch block

Altered T waves

Left axis deviation

estimating the extent and severity of cardiac involvement in DMD. Shortening of the P-Q interval has been found to be particularly valuable in this respect. There is also general agreement that the presence of tall R waves in V_1 is a particularly frequent and consistent abnormality in the disease. A useful measure of this is the algebraic sum of the R and S waves in this lead (that is, [R-S] in V_1), which is abnormal in over 80% of affected boys (see Fig. 6.2). It is particularly interesting that the same abnormality has also been found in up to 10% of female carriers of the disease, whilst it is uncommon in other childhood forms of dystrophy.

The aetiology of the tall R waves in the right precordial lead of the ECG is not clear. Various suggestions have been made, including thoracic deformity, pulmonary hypertension, conduction defect due to myocardial dystrophy, and ventricular septal hypertrophy.

However, none of these suggestions is entirely satisfactory. It seems that this anterior shift of the QRS complex is most probably due to the diffuse interstitial fibrosis in the posterobasal part of the left ventricle (see Fig. 6.3). Involvement of the adjacent papillary muscle would account for mitral valve prolapse that can occur in the disease. Evidence supporting this idea comes from necropsy findings, which have already been discussed, and also from echocardiography. The latter has revealed contraction abnormalities of the left ventricle in most patients, which is first noted in the posterior free wall behind the mitral valve. But why this portion of the myocardium should be so selectively involved in this particular disorder is not clear. Radionuclide imaging suggests a regional metabolic or blood flow alteration.

Cardiac abnormalities are also very frequently observed in patients with BMD. Despite the milder skeletal muscle involvement in BMD, as compared to

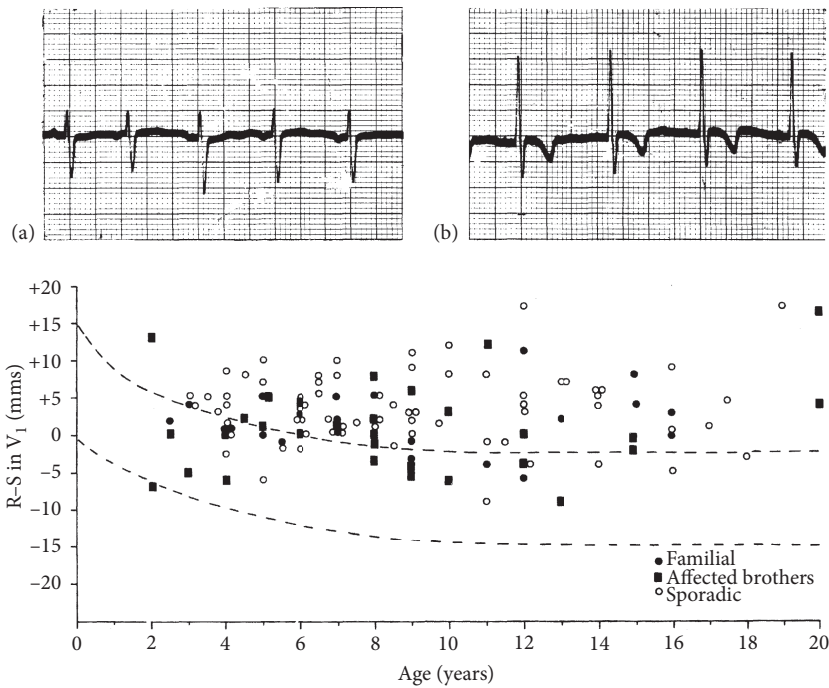


Fig. 6.2 Right precordial lead (V_1) in: (a) a healthy boy and (b) a boy with DMD, both aged 10. Algebraic sum of R-S in V_1 for boys with DMD. Normal 90% confidence limits from Nadas (1963, p. 71).

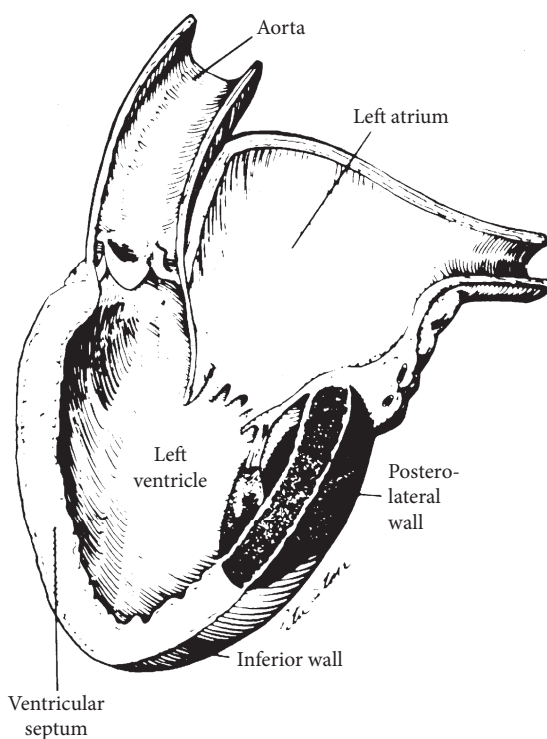
Reproduced from Emery, A. E. H., Abnormalities of the electrocardiogram in hereditary myopathies, *Journal of Medical Genetics*, Volume 9, Issue 1, pp. 8–12, Copyright © 1972, with permission from the author.

DMD, cardiac involvement can be detected in >60% of patients with BMD. Symptomatic cardiomyopathy is, if anything, more common in BMD, compared to DMD, and death from cardiac failure is a well-known complication of this disorder. More recently, XLDCM has been described as an allelic variant of DMD and BMD. In this condition, in which unusual mutations of the dystrophin gene have been documented, the cardiac muscle is severely affected, with features similar to those observed in DMD and BMD, whilst the skeletal muscle is virtually spared. Cardiac transplantation should be offered to these patients with BMD and XLDCM, as the long-term prognosis is generally good.

Whilst ECG is a very useful technique for demonstrating the involvement of the cardiac muscle, the correlation with the severity of the cardiomyopathy is relative poor. In clinical practice, it is recommended to perform cardiac echocardiography every other year in children during the early ambulant stage of

Fig. 6.3 Diagram of the left side of the heart, illustrating the region of selective involvement in DMD patients.

Reproduced from *American Journal of Medicine*, Volume 42, Issue 2, Perloff, J. K., *et al.*, The distinctive electrocardiogram of Duchenne's progressive muscular dystrophy, pp. 179–88, Copyright © 1966, with permission from Elsevier, <<http://www.sciencedirect.com/science/journal/00029343>>.



DMD. After the age of 10 years, or following the loss of ambulation, whichever occurs first, cardiac echocardiography should be performed yearly. Once an echocardiographic abnormality is detected, treatment using angiotensin-converting enzyme (ACE) inhibitors, with or without low doses of β -blockers, is recommended. A study by Duboc and colleagues showed that the ACE inhibitor perindopril had a cardioprotective effect, when given to young DMD boys without evidence of cardiomyopathy (Duboc *et al.* 2007). Prophylactic treatment with ACE inhibitors is not currently routine practice in the UK; however, a randomized, placebo-controlled trial of an 'ACE inhibitor/ β -blocker combination' in young children with DMD is currently under way. Corticosteroids also seem to have a cardioprotective effect; a longitudinal follow-up of boys treated with prednisone showed a delayed onset of cardiomyopathy, compared with historical controls (Markham *et al.* 2008). Echocardiography is the easiest routine test to screen cardiac function, but it is not particularly sensitive and does not detect significant posterobasal left ventricular fibrosis. Cardiac MRI and tissue Doppler echocardiography have better sensitivity and are more likely to detect early left ventricular changes and are preferable for preoperative screening than

standard cardiac echocardiography (Mazur *et al.* 2012). Radioisotope imaging, such as single-photon emission computerized tomography (SPECT) or positron emission tomography (PET), can show defects in left ventricular posterobasal myocardial perfusion in both DMD and BMD, suggesting a vascular component to the development of cardiomyopathy (Quinlivan *et al.* 1996).

Vascular system

Trophic changes in the skin of the extremities of DMD patients are common, especially in the lower limbs and in the later stages of the disease. These changes include coldness and cyanotic mottling and, on occasion, even scleroderma-like changes. Such changes, most probably, stem from inactivity, although Duchenne himself even raised the possibility that muscular dystrophy might have a vascular aetiology. Studies that were designed to determine if the vascular system is involved in DMD focused, perhaps understandably, on muscle vasculature. Several years ago, in Paris, Démos and colleagues (for example, Démos and Maroteaux 1961) measured the circulation time in patients in two ways: arm to arm using fluorescein as a marker, and arm to tongue using sodium dehydrocholate. By subtraction, the 'peripheral circulation time' in the upper limb was determined. In boys with DMD, they found this to be above or below the 95% range for normal children of comparable age and to be significantly reduced in some female carriers. However, there is no defect in the capillary nail bed, and, using venous occlusion plethysmography, one of the authors (Emery) failed to detect any significant changes in total limb blood flow in affected boys at different stages of the disease or in carrier females.

However, more recently, reduced **intramuscular** blood flow has been demonstrated in both the mouse animal model for DMD (the *mdx* mice) and children with DMD. This is believed to be secondary to the absence of nitric oxide synthase (NOS), and hence of nitric oxide (NO), in the skeletal muscle fibres of dystrophin-deficient muscle. As will be discussed later, one of the proteins of the dystrophin-associated glycoprotein complex (DAPC) is neuronal NOS (nNOS). This molecule, physiologically expressed at the periphery of each muscle fibre, is lost from the sarcolemma when dystrophin is also absent. One physiological role of nNOS in skeletal muscle is the production of NO that mediates the inhibition of sympathetic vasoconstriction in contracting muscle. This ability is defective in the mutant mice that lack the gene encoding nNOS, and also in the *mdx* mouse in which nNOS is also secondarily, but severely, deficient. Recently, a similar defect was confirmed in children with DMD. These observations also suggest another mechanism that might contribute to abnormal smooth muscle function in DMD, and eventually to muscle fibre necrosis.

Central nervous system

For some time, there was, perhaps understandably, some reluctance to accept that boys with DMD could also be mentally handicapped. After all, this was yet another misfortune for the affected child and their parents to bear. However, much research, started in the 1960s, confirmed the suspicion of many, first noted in fact by Duchenne, that a proportion of affected boys can have some degree of mental handicap and that, on occasion, this can be severe. It is important to stress that mental retardation is present in only about a third of patients and that highly intelligent youngsters with DMD, who have satisfactorily completed university degree courses, are becoming an increasing reality, in part due to the better opportunity for physically disabled individuals to attend higher degree studies.

Apart from some degree of intellectual impairment, a deficit of memory, autism, ADHD, and behavioural problems have also been reported.

Intelligence quotients of affected boys

There have been a great many studies of intelligence quotients (IQ) in affected boys, the results of which were summarized in the second edition (Emery 1993, p. 116). A total of 721 children were studied in 14 reports, the mean IQ being 82, with 20% having an IQ below 70 and 3% with an IQ below 50 (see Table 6.1). There is therefore considerable variation, ranging from those who are severely handicapped to a few with IQs above 130. In general, the overall mean IQ is about one SD below the normal mean. This reduction in the IQ is not due to any lack of educational opportunity as an result of their physical disability, because it is not found in other diseases with comparable disability such as SCARMD or juvenile SMA. Furthermore, poor educational performance in DMD is often observed early in life when muscle weakness is relatively slight. Whatever causes the intellectual impairment must also operate at an early stage in development, for it is not progressive and does not correlate with duration or severity of the disease. The fact that there is no difference in the IQ between affected boys born to carrier mothers and those who are presumed to be the result of new mutations indirectly confirms a specific association between dystrophin mutations and mental retardation and excludes a maternal factor from being responsible for depressing the IQ.

The most likely explanation is that the depression in the IQ is another pleiotropic effect of the mutant gene. This is supported by the fact that unaffected sibs have normal intellect, and there is often a good correlation between affected brothers. Several investigators have found a high concordance for intellectual function in families with multiple affected children. It is also our experience

Table 6.1 Studies of IQ in boys with DMD ($N = 721$). These figures are based on a large number of published studies (see Emery 1993)

Mean IQ	Range of IQ	Percentage of patients (%) with IQ	
		≤ 70	≤ 50
82	14–134	19	3

Source: data from Emery, A. E. H., *Duchenne muscular dystrophy*, second edition, Oxford University Press, Oxford, UK, Copyright © 1993.

that, whenever an index case is severely mentally handicapped ($IQ < 50$) and has an affected brother, the latter is also severely mentally handicapped. The possibility that there might be bimodality in the distribution of the IQ in affected boys was suggested by some earlier studies, although data collected between the 1960s and 1980s appear to contrast these findings (see Fig. 6.4).

However, recent correlative data on the IQ and site of mutations, that take into account the multiple distal and shorter isoforms, suggest a complex association. In particular, the severity and frequency of mental retardation increases with a progressive loss of functional distal isoforms, and indeed a bimodal distribution of the IQ appears to be confirmed by large studies, in which affected boys were stratified by intragenic deletions (Daoud *et al.* 2009). Interestingly, ADHD has also been shown to be more common in boys, with mutations affecting the brain isoform of the dystrophin isoform Dp140 and Dp71 (Pane *et al.*

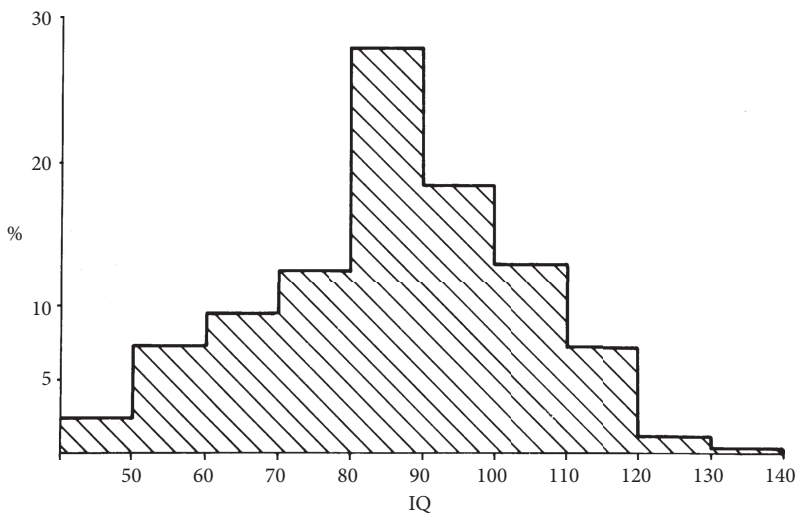


Fig. 6.4 Percentage distribution of IQs in DMD, based on 14 published studies.

2012). As will be discussed in detail in Chapter 9 and as illustrated in Fig. 6.5, the dystrophin locus generates multiple isoforms from internal promoters, located either at the 5' end (the brain, muscle, and cerebellar Purkinje cell isoforms: B, M, and P isoforms) or scattered throughout the gene (see Fig. 6.5). The Dp260 isoform has its first exon in intron 30, Dp140 in intron 45, Dp116 in intron 56, and Dp71 in intron 63 (see Fig. 6.5). An out-of-frame deletion of exon 44 will therefore exert its effect on the three 5' full-length isoforms (B, M, and P) and the Dp260 isoform, whilst the expression of Dp140, Dp116, and Dp71 will be unaffected. In contrast, a deletion extending to exon 50 will also involve the transcription of the Dp140 isoform. A significant contribution of deletions involving the Dp140 isoform to mental retardation has been reported (Bardoni *et al.* 2000). Finally, mutations that affect all dystrophin isoforms are invariably associated with severe mental retardation. This was originally observed by one of the authors (Muntoni) who has reported seven cases with mutations affecting Dp71. Of these, two children never acquired speech and were not testable; the remaining five had moderate to severe mental retardation. In two of these children, a diagnosis of autism preceded the diagnosis of muscular dystrophy. As indicated earlier, larger systematic studies clearly confirm the major role of

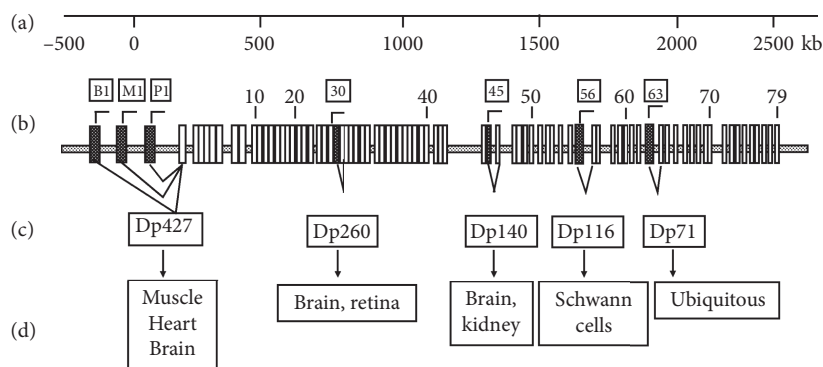


Fig. 6.5 Schematic representation of the dystrophin gene. (a) The size occupied by the dystrophin gene is indicated in kilobases (kb). (b) The locations of the various first exons and promoters are indicated on the top of each of the vertical bars representing the dystrophin exons, whilst (c) indicates the names of the resulting isoforms. In (b), B1, M1, and P1, respectively, indicate the brain, muscle, and Purkinje cell promoters, which are located before exon 2 and encode three full-length isoforms. The promoter located in intron 30 encodes the Dp260 isoform, whilst the one in intron 45 encodes the Dp140 isoform. The promoters located in introns 56 and 63 are responsible for the transcription of the Dp116 and Dp71 isoforms, respectively. (d) Tissues in which the various isoforms are preferentially expressed.

Dp71 on mental function (Daoud *et al.* 2009). For a comprehensive review on dystrophin and the brain, refer to Mehler (2000).

Partition of intelligence quotients

Several studies have been made to determine what aspect of intellect may be especially affected in DMD, by comparing performance and verbal IQs (see Table 6.2). In most studies published, the verbal IQ is more affected, the overall difference from the performance IQ being about 5–8 points.

The impairment of verbal ability seems to be due to a defect in memory for patterns, numbers, and verbal labels, implying a particular deficit in memory function. Some depression of the verbal IQ has also been found in BMD, but not in LGMD or facioscapulohumeral muscular dystrophy.

Relationship between degree of muscle weakness and cognitive involvement

In a study performed over a large number (580) of DMD cases followed at Hammersmith Hospital, we found that the early motor milestones and the acquisition of ambulation were inversely correlated with mental function, that is, the more severe the mental retardation, the greater the delay in the early motor milestones. On the whole, however, DMD patients with severe mental retardation were not more severely affected than those without mental retardation (personal observation from Muntoni). Other studies have, in fact, suggested that DMD children with severe mental retardation seem to be less severely affected. In affected boys with severe mental handicap (IQ <50), the ages at onset and at becoming confined to a wheelchair were somewhat later (see Table 6.3), and the fall in SCK levels with age was less marked.

Behavioural and emotional disturbances

Behavioural and emotional disturbances have been commented upon by a number of investigators and might stem from a sense of failure, frustration, and

Table 6.2 Performance (P) and verbal (V) IQ in boys with DMD, calculated from data given in eight published studies (full references can be found in Emery 1993, p. 118)

Number of cases	Mean IQ		P minus V
	P	V	
299	90.65	85.21	+5.44

Source: data from Emery, A. E. H., *Duchenne muscular dystrophy*, second edition, Oxford University Press, Oxford, UK, Copyright © 1993.

Table 6.3 Age (years) at onset and at becoming confined to a wheelchair in patients with normal intelligence or with severe mental handicap

Age (years)						p	Reference
Normal intelligence			Severe mental handicap				
N	Mean	SD	N	Mean	SD		
Age at onset (years)							
15	2.20	0.78	15	2.43	1.03	NS	Emery <i>et al.</i> 1979
29	3.64	1.72	10	3.61	2.01	NS	Bortolini and Zatz 1986
Age at becoming chairbound (years)							
12	8.77	1.12	13	9.65	1.60	NS	Emery <i>et al.</i> 1979
24	9.49	1.70	11	10.83	1.65	<0.05	Bortolini and Zatz 1986

N, number; NS, not significant; p, probability; SD, standard deviation.

Source: data from Emery, A. E. H. *et al.*. A study of possible heterogeneity in Duchenne muscular dystrophy, *Clinical Genetics*, Volume 15, Issue 5, pp. 444–9, Copyright © 1979; and Bortolini, E. R. and Zatz, M., Investigation on genetic heterogeneity in Duchenne muscular dystrophy, *American Journal of Medical Genetics*, Volume 24, Issue 1, pp. 111–17, Copyright © 1986.

distress, generated by the progressive physical disability. However, in view of the nature of the disorder, it is perhaps surprising that the majority of boys are not emotionally disturbed, and yet they are not. Nevertheless, allowing for age and IQ, boys with DMD do have a higher incidence of emotional disturbances than other physically handicapped children without cerebral involvement, and it is just possible that this too could represent part of the disease. The assessment by one of us (Muntoni) of a large series of patients at Hammersmith Hospital suggests that there might be a small increased risk of epilepsy in children with DMD and BMD. Of 254 boys with these conditions (201 DMD and 53 BMD), eight children (4 DMD and 4 BMD) had confirmed epilepsy, equivalent to a total incidence of 3.14% (with a subgroup incidence of 1.99% in the DMD group, and 7.54% in the BMD group). A more recent study of 200 DMD patients has confirmed an increased risk of epilepsy, affecting 6.3% of patients, compared with 1% of the general paediatric population; interestingly, there was no correlation between epilepsy and the presence of learning difficulties (Pane *et al.* 2013).

Neurological investigations

The head circumference appears to be significantly greater than normal, but there is no correlation between the head size and intellectual performance. However, the rare patients with severe mental retardation and mutations affecting the expression of Dp71 tend to have a normal, or slightly less than normal, head circumference. Electroencephalography (EEG) has been reported as normal in a carefully controlled and blind study by Barwick *et al.* (1965). In some other studies, however, up to a half of the records have been considered abnormal in some non-specific way. Nevertheless, many patients with apparently abnormal EEGs have had normal IQs. In any case, no specific EEG abnormality has been detected in the disease.

CT has been used to study central nervous system involvement. Yoshioka and colleagues in 1980 found evidence of slight cerebral atrophy in two-thirds of the 30 cases they examined, and the older the patient, the more severe the atrophy. There were many with a low IQ among those with cerebral atrophy, but, in those with apparently normal CT findings, only three had a low IQ. Abnormal CT findings therefore seem to be associated with a low IQ. MRI, however, has revealed no significant abnormality, apart from mild cerebral atrophy in some cases.

From a pathological point of view, two studies have been performed on the brain of children with DMD. In 1966, Rosman and Kakulas examined the brain of seven DMD cases (at least two of these could have been BMD). In all cases with a mental defect, they found microscopic heterotopias in the cerebral cortex. However, in a later, more extensive study of 21 cases of classical DMD, Dubowitz and Crome (1969) could detect no gross pathological abnormality. Nevertheless, recent detailed microscopic studies have revealed abnormal dendritic development and arborization, at least in some cases, and this may underlie the intellectual impairment. Dystrophin has been found to be absent in the brain of a boy with DMD. Recent immunocytochemical studies have shown that dystrophin is selectively localized to post-synaptic neurons in the cerebral cortex, hippocampus, and cerebellum and co-localizes with γ -aminobutyric acid (GABA)-A receptor subunit clusters in these regions. In the *mdx* mice, a marked reduction in the number of clusters immunoreactive for the $\alpha 1$ and $\alpha 2$ GABA subunits was observed in the cerebellum and hippocampus. These data suggest that dystrophin may play an important role in the clustering or stabilization of GABA-A receptors in a subset of central inhibitory synapses (Knuesel *et al.* 1999). Quantitative differences in the nicotinic receptors in the hippocampus (Ghedini *et al.* 2012) and in the GABAergic transmission in the amygdala (Sekiguchi *et al.* 2009) have also been reported in the *mdx*. The authors concluded that these deficits may be related to the cognitive impairment observed in DMD children.

Though affected boys have normal visual acuity, electroretinography has revealed significant defects in a number of cases. Furthermore, dystrophin has been localized to the outer plexiform layer of the normal retina, and a defect in the retinal isoform in DMD (Dp260) (see Fig. 6.5) may account for the observed retinographic changes and also indicates that dystrophin may well play a role in retinal neurotransmission.

Skeletal system

A number of skeletal changes have been observed in DMD. They include progressive narrowing of the shafts of the long bones due to a reduction in the size of the medullary cavity and later thinning of the cortices. Since, at the same time, the head remains more or less the same size, the long bones assume a characteristic 'dumb-bell' appearance (see Fig. 6.6). There is often impaired development of the pelvic bones and scapulae, and various skeletal deformities occur, including lumbar lordosis, scoliosis, and coxa valgus. The bones themselves undergo progressive rarefaction and decalcification, beginning at the ends of the long bones.

For a long time, these changes were thought to be a direct consequence of a genetic defect, and such terms as 'bone dystrophy' and 'osteomyopathy' were used. However, it is now quite clear that these same changes can occur in any disorder associated with prolonged immobility. They are not due to an associated genetic factor, but to disuse, to the absence of the normal stresses and strains imposed by muscular attachments, and to the adoption of abnormal

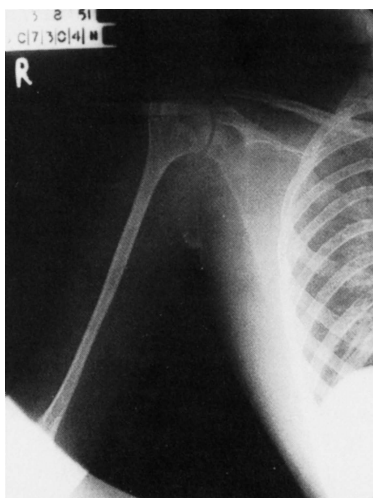


Fig. 6.6 Marked osteoporosis of the humerus in an advanced case of DMD.

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postures of the body and positions of the limbs, as a consequence of muscle weakness and contractures.

Bone densitometry data, obtained with dual-energy X-ray absorptiometry (DEXA) scanning, have confirmed that children with DMD are significantly osteopenic. Ambulant children with DMD have a significantly decreased bone density, as early as 5–7 years of age. However, children with other conditions, such as LGMD, have a similar decrease in bone density, suggesting that this problem is secondary to reduced loading and immobility, rather than a pleiotropic effect of the defective gene. It is also interesting to note that bone mineral density actually increases in young children treated with corticosteroids, presumably because the child is more active. Later on, however, corticosteroid-induced osteoporosis develops.

Other manifestations

Despite the expression of dystrophin in the cochlear hair cells, hearing is not affected (Dodson *et al.* 1995). Thymus hyperplasia has been reported by several authors, the relevance of which is not at all clear. Puberty is often delayed, and this problem is compounded now by the use of corticosteroids. Hyperoestrogenaemia may also occur, and obesity is frequent, again more frequently in boys treated with corticosteroids. However, apart from the study of skeletal muscle, cardiac muscle, and the central nervous system, it has to be admitted that there have been few recent systematic investigations of other systems, organs, or tissues.

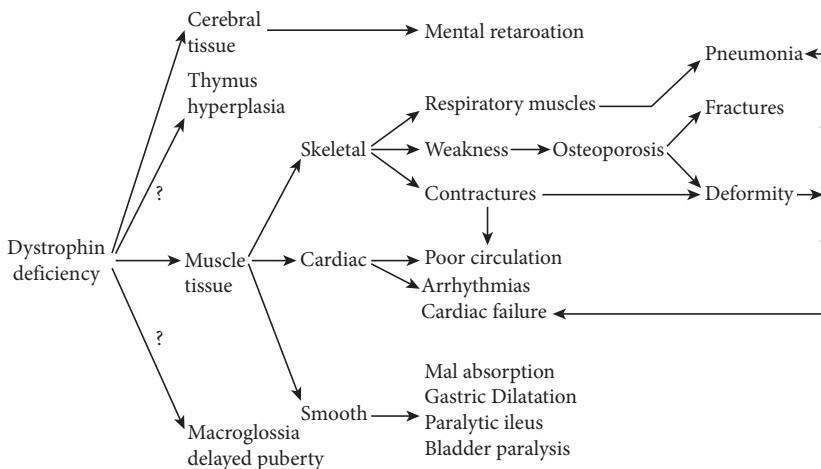


Fig. 6.7 Pleiotropic effects of the Duchenne gene.

With what is known so far, it is possible to construct a simple diagram of the pleiotropic effects of the Duchenne gene (see Fig. 6.7). Some of the abnormalities found in the disease relate directly to skeletal, cardiac, and smooth muscle involvement. Others are, at present, more difficult to relate to the primary protein (dystrophin) defect in the disease.

Summary and conclusions

Most of the clinical features of DMD stem from the involvement of skeletal muscle. However, there is increasing evidence that other tissues may also be directly affected by the disease.

Smooth muscle of the gastrointestinal tract, and perhaps the bladder, may be affected, and there is overwhelming evidence of cardiac muscle involvement, myocardial fibrosis being an important feature. This particularly affects the posterobasal portion of the left ventricle and accounts for the ECG changes of tall R waves in the right precordial lead that are already evident in the early ambulant phases of the disorder. The vascular system does not appear to be significantly affected, although a mild reduction of blood flow, following exercise, related to the deficiency of NOS has now been documented. There is clear evidence of a defect in cerebral function, which is a direct consequence of the genetic defect, most obviously expressed in a lowered IQ which, on average, is roughly one SD below the normal mean, the verbal IQ being more affected than the performance IQ. A pattern of a more severe loss of IQ, with or without ADHD, with more distal mutations is recognized. The skeletal system is only secondarily affected by disuse atrophy, and, so far, there is no convincing evidence that any other tissues or organs are directly affected by the genetic defect.

The various clinical manifestations of DMD are now beginning to be related to the structure and functioning of the dystrophin gene in different tissues. There is much scope for research in relating the phenotype to molecular events in this disease.

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Biochemistry of Duchenne muscular dystrophy

Introduction to the biochemistry of Duchenne muscular dystrophy

The literature on the biochemistry of muscular dystrophy is overwhelming, and many biochemical abnormalities have been reported. It could be argued that, now that the primary defect has been identified in DMD and shown to be a deficiency of muscle dystrophin (see Chapter 9), it is irrelevant to approach an understanding of the pathogenesis through the findings of conventional biochemistry. We do not share this view but feel that molecular and biochemical studies could complement each other. What has been learned so far concerning biochemical changes in dystrophic muscle and how these relate to the deficiency of dystrophin will doubtless fill in details of how the disease process starts and progresses, and why it affects some muscles more than others. It is also conceivable that the more we know of the detailed pathogenesis of DMD, the more we may understand these processes in other muscular dystrophies. And, with such details, it may be possible to better consider a rational approach to any drug therapy.

Selection of material, patients, and controls

One important problem is the selection of appropriate material for study. Muscle is clearly the obvious choice, but there is then the problem of assessing the significance of any changes that could be secondary to the disease process. There is also the very serious ethical and practical problem of obtaining material for study, and, in practical terms, this is limited to the diagnostic material. The stage of the disease is also important, for clearly abnormalities found early in the course of the disease are more likely to be closer to the basic biochemical defect.

The choice of appropriate controls is also a problem, for the diagnosis is usually established in an affected boy at around 3–5 years of age. Appropriate muscle tissue, usually the quadriceps, from a normal boy of this age is not too easily acquired. Also, the use for comparison of muscle tissue from other neuromuscular

disorders is questionable, unless the research question is to establish whether or not any abnormality is specific to DMD.

Molecular basis

When the gene responsible for a particular disorder can be isolated, cloned, and sequenced, it is possible to see what the gene synthesizes. This has sometimes been referred to as 'reverse genetics' (or positional cloning), for, in the past, it was necessary to start by identifying the product of the defective gene, but now it is possible to identify the mutant gene first and then determine its product. This has been successfully achieved in DMD where the primary genetic defect has been shown to be a deficiency of dystrophin (see Chapter 9).

Muscle tissue

There have been many excellent reviews of earlier reported biochemical abnormalities in DMD, and a full reference list of these can be found in previous editions of the book. It would be impossible to review all the abnormal findings that have been reported, nor would this be valuable. Instead, the discussion will concentrate on those that have been found in early cases of the disease, and preferably that have been confirmed in several different laboratories.

The various biochemical changes that have been observed in affected muscle can be conveniently considered as being the result of wasting, invasion by other tissue elements, and 'dedifferentiation'.

Muscle wasting

As the disease progresses, so functioning muscle tissue degenerates and is gradually replaced by fat and connective tissue. If the results are expressed in terms of total muscle weight, then particular constituents may appear to be reduced when, in fact, the levels in functioning muscle tissue may be normal. A solution to this problem is to express results in terms of some specific reference base. In the past, this has been total protein or, better still, non-collagen protein, which corresponds to that fraction of the total muscle protein that is soluble in dilute alkali. In health, the amount of non-collagen protein (expressed as non-collagen nitrogen) is roughly the same in different skeletal muscles (see Table 7.1), and, at least in later childhood and young adulthood, it is not significantly affected by age or sex. Non-collagen protein represents over 90% of the total protein of normal muscle, but it may be <50% in severely affected dystrophic muscle.

Myosin, or some similar contractile protein, has also been recommended as a reference base. Whereas fibroblasts, macrophages, lipocytes, and other cells present in dystrophic tissue might contribute to non-collagen protein, they

Table 7.1 Non-collagen nitrogen (NCN), expressed as mg (g of wet weight)⁻¹, in various normal human skeletal muscles (unpublished data)

Muscle	Number	NCN	
		Mean	SD
Rectus abdominis	20	23.3	6.2
Gastrocnemius	8	26.7	5.5
Deltoid	6	20.7	2.7
Pectoralis major	10	23.9	5.6
Miscellaneous*	9	24.0	3.6
Total	53	23.7	5.3

* Quadriceps (3); sternomastoid (2); sartorius (1); transversalis (1); diaphragm (1); latissimus dorsi (1).

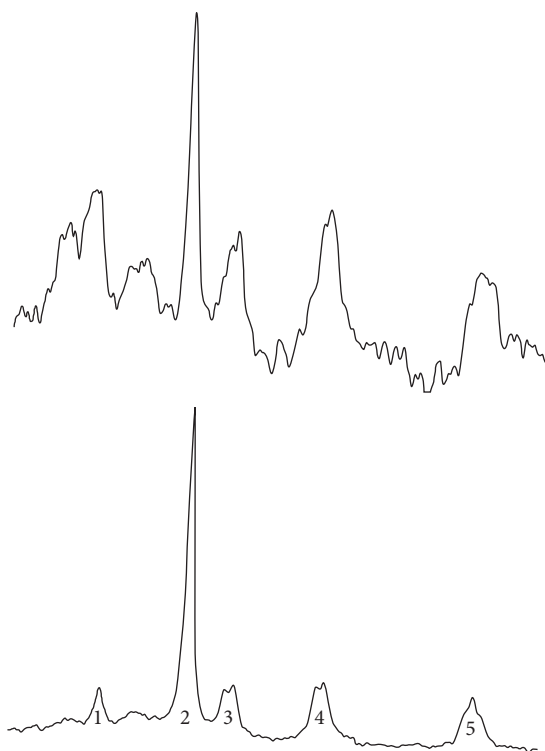
would not affect the myosin content. As would be expected, as dystrophic muscle degenerates, so its myosin content decreases. When levels of ATP and creatine phosphate are expressed in terms of myosin, they are no different from normal. This result is in stark contrast to several earlier studies that reported reduced levels using non-collagen protein as the reference base. Normal levels of ATP have also been confirmed by the technique of nuclear magnetic resonance (NMR). The fact that ATP levels are normal has important implications. It means that energy stores for muscle contraction are adequate, at least early in the course of the disease, and therefore this is not the cause of muscle weakness. The latter seems more likely to be a reflection of the loss of muscle fibres due to their degeneration.

However, studies using NMR spectroscopy seem to indicate that creatine phosphate may be reduced (see Fig. 7.1).

As the amount of functioning muscle tissue decreases, this has several other consequences. Occasionally, the glucose tolerance curve may be mildly abnormal, due to an inadequate disposal of glucose associated with the reduced muscle mass, and plasma free fatty acids may be raised. Glucose and lipid metabolism has been studied in detail, and the abnormal energy metabolism observed is attributed to either a calorie shortage or, more probably, muscle degeneration. More importantly, changes occur in creatine and creatinine metabolism, changes that have also been recognized for many years. Creatine is largely synthesized in the liver and is delivered to the skeletal muscle where it is converted to creatinine, which readily diffuses into the circulation and is excreted in the urine. In fact, the amount of creatinine excreted each day by any individual is remarkably constant and is roughly proportional to the total body muscle mass.

Fig. 7.1 Magnetic resonance spectra of the gastrocnemius muscle in a control (below) and a 15-year-old boy with DMD (above). In the affected boy, there is a significant increase in intracellular pH (normal 7.01, affected 7.28) and an apparent reduction in the ratio of creatine phosphate to inorganic phosphate. Peaks: 1, inorganic phosphate; 2, creatine phosphate; 3–5, ATP.

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In general terms, as muscle wastes from whatever cause, so the level of creatine in the plasma, and especially the urine, will increase, and the amount of creatinine in the urine will decrease. These changes, however, appear to be somewhat removed from the basic defect in dystrophy, not only because they are not specific, but also because no abnormalities in creatine and creatinine excretion occur in female carriers of the disease, unless they have significant muscle weakness (Emery 1963). Often female carriers investigated, in only one, who was a manifesting carrier with marked muscle weakness, was the creatine/creatinine ratio abnormally high (see Fig. 7.2).

As skeletal muscle degenerates, various breakdown products will be released and appear in the urine. For example, 3-methylhistidine is a known constituent of both actin and myosin, and, as muscle breaks down, the concentration in muscle decreases, and, when expressed in terms of creatinine, the urinary excretion increases. In fact, the urinary excretion of 3-methylhistidine is an excellent measurement of myofibrillar protein catabolism. Thus, whilst creatinine excretion may be taken as an index of total muscle mass, 3-methylhistidine excretion is an index of muscle breakdown.

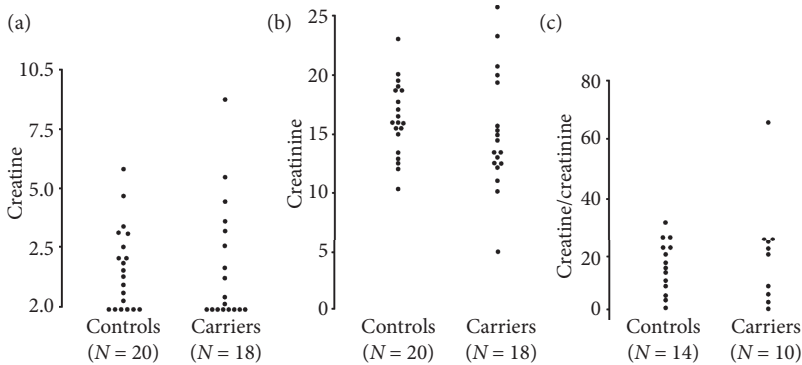


Fig. 7.2 The urinary excretion of (a) creatine, (b) creatinine (mg/kg/24 hours), and (c) ratio of creatine/creatinine ($\times 100$) in healthy women and DMD carriers (unpublished data).

Carnitine is largely synthesized in the liver and kidneys and is subsequently taken up by cardiac and skeletal muscle. As muscle breaks down, so carnitine is released, and concentrations in dystrophic muscle are significantly reduced. Since carnitine is an important co-factor in fatty acid oxidation, the reduction in muscle carnitine might also explain the accumulation of long-chain fatty acid derivatives in this tissue.

In addition to the proteins released during various stages of muscle breakdown, a general dysregulation of vesicle trafficking has been demonstrated in cultured dystrophic myocytes (Duguez *et al.* 2013). The authors have hypothesized that the disturbance of the export of proteins through vesicles is independent from the myonecrotic cascade and contributes to the pathophysiology of DMD.

Finally, although there is no doubt that many proteins are lost from dystrophic muscle, there is also evidence that some substances may actually enter affected muscle fibres. Experimentally, ingress of horseradish peroxidase (MW 40 000 Da) and Procion® yellow (MW 674 Da) has been demonstrated, and evidence suggests that calcium, immunoglobulin (Ig) G, complement, and albumin enter affected muscle fibres (see Chapter 10). Of particular interest is the finding that calcium and albumin enter fibres lacking dystrophin in female carriers of X-linked dystrophy. This has important implications for the pathogenesis and will be discussed later (see Chapter 10).

The abnormal plasma membrane permeability in dystrophin-deficient muscle can also be assessed *in vivo* in the animal models of muscular dystrophy, using tracer molecules such as Evans blue. This low MW diazo dye does not cross into skeletal muscle fibres in normal mice. In contrast, *mdx* mice showed significant Evans blue accumulation in skeletal muscle fibres.

The Evans blue accumulation is significantly increased by strenuous physical exercise, providing some evidence for the exercise-induced damage of muscle fibres (Straub *et al.* 1997).

More recently, an albumin-targeted contrast agent (MS-325) was used *in vivo* to study the sarcolemmal integrity of *mdx* mice by MRI. Intravenously injected MS-325 does not enter skeletal muscle of normal mice. However, *mdx* mice showed significant accumulation of MS-325 in skeletal muscle. The results indicate that it is possible to use this non-invasive technique for evaluating the localization and severity of skeletal muscle damage in muscular dystrophy.

Enzyme changes in dystrophic muscle

There is general agreement that glycolysis, as well as the activities of most individual glycolytic enzymes, is reduced in muscle from DMD patients, as well as in several other dystrophies. Ellis's (1980) detailed studies indicate that, in dystrophic muscle, fructose is incorporated into the glycogen pathway, at the expense of glucose, and this results in increased lipogenesis. Fatty acid oxidation is also reduced, but again this is not specific to DMD.

A great many individual enzymes have been studied in muscle tissue from patients with DMD. In those instances where enzyme levels have been expressed in terms of non-collagen protein and studies made specifically on DMD, some general conclusions can be made. The level of activity of some enzymes appears to be normal, at least in the early stages of the disease (see Box 7.1).

Other enzymes, however, have reduced activity (see Box 7.2), in some cases even from very early on in the disease process, as in the case of AMP deaminase. Interestingly, a deficiency of the erythrocyte form of phosphofructokinase is not associated with muscle disease but results in a non-spherocytic haemolytic anaemia.

The reduced activity of these various enzymes is largely the result of efflux from diseased muscle fibres, though this cannot be the entire story. Thus, adenylate kinase, which has a relatively low MW (21 000 Da), is reduced in affected muscle but is not increased in serum (see Chapter 4), and this is also true of AMP deaminase. On the other hand, the aminotransferases are not significantly reduced in affected muscle but are increased in serum. Finally, acylphosphatase is one of the smallest enzyme molecules known (9400 Da) and is abundant in skeletal muscle, largely in the soluble sarcoplasm, yet there is an apparently normal activity in affected muscle. The explanation for these apparent contradictions may lie in the relative rates of synthesis (perhaps influenced, to some extent, by physical activity) versus the destruction of different enzymes

Box 7.1 Enzymes with normal activity in skeletal muscle tissue in DMD

Aminotransferases (GPT, GOT)
 Succinic dehydrogenase
 Hexokinase
 Phosphohexose isomerase
 Aconitase
 Cytochrome oxidase
 Alkaline phosphatase
 Acylphosphatase
 Fructose-1,6-diphosphatase
 Lysolecithin phospholipase
 Superoxide dismutase
 Methylthioadenosine nucleosidase
 Adenylosuccinase
 Monoamine oxidase
 Glyoxalase II

Box 7.2 Enzymes with reduced activity in skeletal muscle tissue in DMD

Phosphoglucomutase
 Phosphofructokinase
 Aldolase
 Triosephosphate isomerase
 Phosphoglyceraldehyde dehydrogenase
 Phosphoglycerate kinase
 Enolase
 PK
 LDH
 Fumarase
 Glycogen phosphorylase
 Glycogen synthetase
 CK
 AMP deaminase
 Adenylate kinase

Box 7.3 Enzymes with increased activity in skeletal muscle tissue in DMD

Glucose-6-phosphate dehydrogenase
6-phosphogluconate dehydrogenase
Isocitrate dehydrogenase
Malate dehydrogenase
5'-nucleotidase
Ribonuclease
Glutathione reductase
Prote(in)ases
Carnitine palmityltransferase
Lipid peroxidation
Phosphodiesterases

in affected muscle fibres, as well as their clearance rates from plasma. So far, however, very little is known of the relative importance of these different factors for individual enzymes.

Finally, and perhaps more interestingly, the activity of some enzymes is actually increased in DMD (see Box 7.3). These changes are attributable to the invasion of affected muscle by macrophages and fibroblasts, as well as to the necrosis of affected muscle fibres. Macrophages and fibroblasts are known to contain several nicotinamide adenine dinucleotide phosphate (NADP)-linked dehydrogenases (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase) and other enzymes such as 5'-nucleotidase and ribonuclease. These cells also contain a number of proteases, including cathepsins, lysosomal acid hydrolases, and calcium-activated proteases, which are all increased in dystrophic muscle. These enzymes attack and break down muscle protein, and their increase is probably also an adaptive response of the muscle fibre to its degeneration and necrosis.

It should be noted, however, that this division into enzymes that are normal, reduced, or increased in dystrophic muscle, although convenient, is somewhat arbitrary, because it often depends on what stage in the disease process the assays are carried out. In almost all cases, the activity is normal at the beginning, and abnormally low or high levels are found only later in the course of the disease. But some, such as acylphosphatase seem to remain at more or less normal levels, right until the very late stages of the disease.

Membrane enzymes

Many muscle enzymes are free in the sarcoplasm, but some are attached to membranes such as the sarcoplasmic reticulum. The latter include adenylate cyclase, guanylate cyclase, and Ca^{2+} -, (Na^+ and K^+)-, and Mg^{2+} -ATPases. Until recently, enzyme studies have been limited to whole muscle homogenates, which, at least later in the disease, are contaminated by extraneous adipose and connective tissue. It has therefore not been possible to study the activity in isolation of those enzymes that are attached specifically to muscle membranes. To circumvent this problem, minimally affected muscle should be studied, and the technique of using 'skinned fibres' has been developed. In these preparations, the surface membrane of the muscle fibre is removed mechanically or disrupted chemically. Using these techniques, it seems that, in the early stages of the disease, the sarcoplasmic reticulum and contractile protein functions are normal, as is the calcium uptake by the sarcoplasmic reticulum. The study of skinned fibres has also shown that, in DMD, despite the deficiency of dystrophin, these contract normally, and therefore the myofibrils must themselves be intrinsically normal.

'Dedifferentiation'

A number of observations indicate that, in many ways, dystrophic muscle resembles fetal muscle, for which the term 'dedifferentiation' has sometimes been used. First, it is less easy to distinguish different histochemical fibre types in dystrophic muscle, which is also a feature of fetal muscle (see Fig. 7.3), even at term. Second, certain phospholipid changes in dystrophic muscle (more sphingomyelin, less lecithin plus choline plasmalogen, and more total cholesterol) are very similar to those found in fetal muscle. Third, fetal myosins are found in muscle from patients

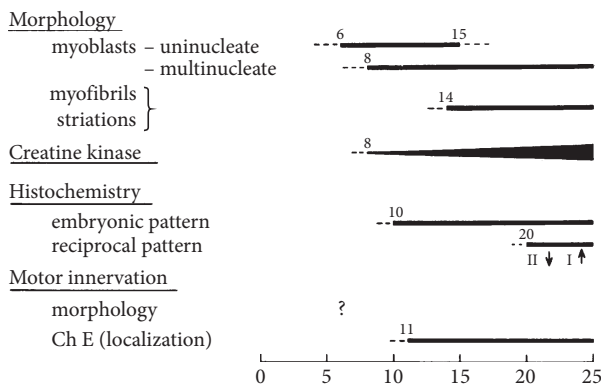


Fig. 7.3 Times (weeks of gestation) at which various aspects of muscle development become apparent. ChE, choline esterase.

with DMD, but also in other conditions characterized by muscle damage and regeneration, congenital myopathies, and SMA. Fourth, another fetal isoform of muscle protein, re-expressed in regenerating muscle, is cardiac troponin T.

Finally, and most intriguingly, the isoenzyme patterns of dystrophic muscle resemble those of fetal muscle, rather than adult muscle. This was first shown in the case of LDH. This enzyme is composed of five isoenzymes, each being formed by the tetrameric association of two subunits, synthesized by two separate genes, referred to as M and H (see Fig. 7.4). The M subunit predominates in adult skeletal muscle, and the H subunit in cardiac muscle.

On electrophoresis, the most rapidly migrating isoenzyme LDH-1 has the composition H_4 ; LDH-2, H_3M ; LDH-3, H_2M_2 ; LDH-4, HM_3 ; and LDH-5, M_4 . The amounts and proportions of the M and H subunits (LDH-M, LDH-H) can be determined in a number of ways. The proportions of LDH-M and LDH-H in some normal skeletal muscles are given in Table 7.2.

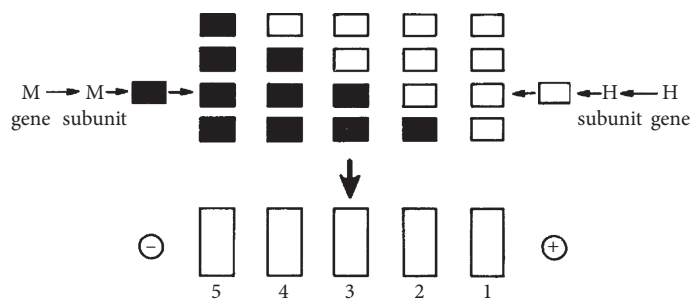


Fig. 7.4 The formation of the five isoenzymes of LDH.

Table 7.2 Proportions of LDH-M and LDH-H in various normal skeletal muscles*

Muscle	Number	Proportion (%)	
		LDH-M	LDH-H
Rectus abdominis	5	87.0	13.0
Diaphragm	1	89.2	10.8
Gastrocnemius	3	86.7	13.3
Quadriceps	1	89.6	10.4
Latissimus dorsi	1	87.4	12.6
Pectoralis major	6	87.9	12.1
Deltoid	2	95.6	4.4
Soleus	2	67.1	32.9

* Unpublished data.

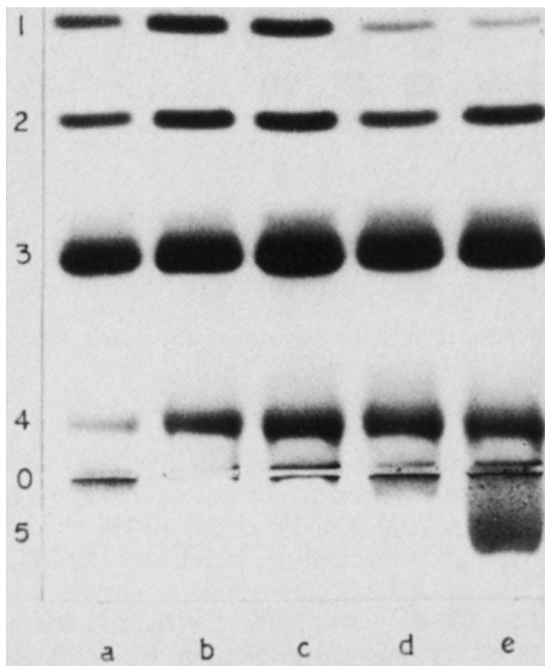


Fig. 7.5 LDH isoenzyme patterns in muscle extracts from: (a) a 3-year-old boy with preclinical DMD; (b) a 400 g fetus; (c) a 7-month stillbirth; (d) a neonate; (e) a 3-month-old normal infant. 0 is the origin, and the anode is at the top.

Although there are some variations in different skeletal muscles, LDH-M clearly predominates. However, in fetal skeletal muscle, LDH-H predominates, and the isoenzyme pattern resembles that seen in DMD, even in the preclinical phase (see Fig. 7.5). It may be that the normal adult pattern is never attained, in which case the term ‘dedifferentiation’ is hardly appropriate. Incidentally, there does not appear to be a complete absence of LDH-5 in all cases of DMD. A reduction in LDH-M is also found in some female carriers, but the change is not specific to DMD but is also found in a number of other neuromuscular disorders. Analogous changes in isoenzyme patterns have also been reported for CK, aldolase, isocitrate dehydrogenase, malate dehydrogenase, adenylate kinase, and enolase.

The implication of these various findings is that dystrophic muscle fibres due to the stochastic events leading to fibre necrosis, often sectorial within an individual muscle fibre, are at different stages of developmental progress and synthesize polypeptides normally produced only during fetal development, not post-natally. Indeed, using immunohistochemical techniques, the re-expression of fetal-specific myosins has also been localized to regenerating fibres in DMD,

whilst neonatal isoforms are still expressed even in fibres of normal size, indicating a relatively recent history of regeneration. Other proteins, such as the enzyme hypoxanthine-guanine phosphoribosyltransferase, are also significantly increased in muscle in patients with DMD even from the age of 2, again suggesting increased protein synthesis and regenerative activity.

Despite these detailed studies, the mechanism of progressive muscle fibre necrosis in dystrophin-deficient muscular dystrophies is not fully understood. The answer to the fundamental question as to whether a deficit of muscle energy production plays a relevant role in the cascade of events, starting with dystrophin deficiency and ending with muscle fibre degeneration, is also not known. Magnetic resonance spectroscopy (MRS) data obtained *in vivo* have added further evidence, suggesting that specific and significant metabolic abnormalities can be identified in the dystrophin-deficient skeletal muscle. Furthermore, gene and protein profiling data, obtained from the muscle of DMD children and *mdx* mice, provide additional evidence of the importance of the secondary metabolic imbalance observed in the dystrophin-deficient muscle, with the reduced transcription of many genes involved in energy metabolism, and also profound disturbances in genes and proteins involved in the inflammatory response, extracellular matrix remodelling, and calcium homeostasis. Temporal analysis of these pathways appears to indicate stage-specific remodelling of DMD muscle, with the activation of inflammatory pathways predominating in the pre-symptomatic stages and the marked activation of fibrotic pathways (that is, transforming growth factor (TGF)- β), and failure of metabolic pathways later in the disease (Chen *et al.* 2005). These latter data will be discussed in detail in Chapter 10.

Regarding MRS studies, the skeletal muscle oxidative and non-oxidative energy production has been analysed, both in DMD and BMD muscle, *in vivo* (Lodi *et al.* 1999). The main findings are that, following aerobic exercise, the dystrophin-deficient muscle acidifies less than normally, suggesting a deficit in the glycolytic lactate production as a cause of higher end-exercise cytosolic pH in patients. The interpretation of these findings *in vivo* as being due to a glycolytic defect is consistent with a number of observations *in vitro* of altered glucose metabolism in dystrophin-deficient muscle. The glucose transporter GLUT4, which co-localizes with dystrophin and other dystrophin-associated proteins, including nNOS on the inner sarcolemmal membrane, is reduced in the diaphragm and the heart of *mdx* mice to ~50% of the normal value. Furthermore, nNOS is also significantly decreased in dystrophin-deficient skeletal muscle, and this might contribute to the deficit in the glycolytic lactate production in dystrophin-deficient muscle, as nNOS increases the rate of glucose transport and metabolism in skeletal muscle.

Whether and how the glycolytic deficit in dystrophin-deficient muscle contributes to fibre necrosis is difficult to establish. It is, however, possible that, during exercise, the glycolytic deficit may be responsible for a greater ionic imbalance and that this may be related to chronic skeletal muscle injury.

Cultured myoblasts

The study of myoblasts in tissue culture, free from all the possible confounding effects of extraneous factors, would seem to offer the ideal system for studying DMD. Unfortunately, there are technical difficulties to be overcome in this approach, not least of which is the presence of other cell types (mostly fibroblasts) that 'contaminate' such cultures. This is a problem particularly with primary explants where the biopsy material is first freed of any obvious fat and connective tissue, and then small fragments of about 1 mm in size are grown in culture vessels with appropriate nutrient medium, usually enriched with chick embryo extract or fetal calf serum. To avoid problems of possible contamination with fibroblasts, cellular outgrowths from explants can be dissociated, and the dissociated cells then transferred to secondary monolayer cultures, a procedure that can be repeated. In this way, we chose to study fetal muscle, in which fibroblast contamination is minimal in any event (see Fig. 7.6). A dissociation technique, coupled with special culture conditions, can also be used, which ensures that growth of myoblasts is encouraged, at the expense of other cell types.

As a further refinement, clonal cultures can be set up, whereby the progeny of single myoblasts can be studied; myoblasts can also be immortalized, using viral vectors. Finally, muscle-nerve co-cultures (for example, DMD muscle and rodent spinal cord) can be used to study the possible effects of innervation *in vitro*.

Whether muscle is cultured aneurally or innervated, no gross morphological abnormalities are evident in DMD. Detailed scanning electron microscopic studies, however, have revealed changes in cell surface morphology, which could explain the low adhesiveness and delayed fusion of dystrophic myoblasts in culture, and these abnormalities are expressed maximally after myoblast fusion. Some reported biochemical abnormalities have been interpreted as indicating that dystrophic muscle in culture reaches a lesser degree of maturity than normal muscle. For example, in dystrophic muscle culture, the CK BB isoenzyme is significantly increased (and the CK MM decreased), although this is not specific to DMD. The protein degradation rate in cultured Duchenne muscle cells is normal, which supports the idea that the loss of contractile muscle proteins in the disease is largely the result of reduced synthesis, rather than increased degradation.

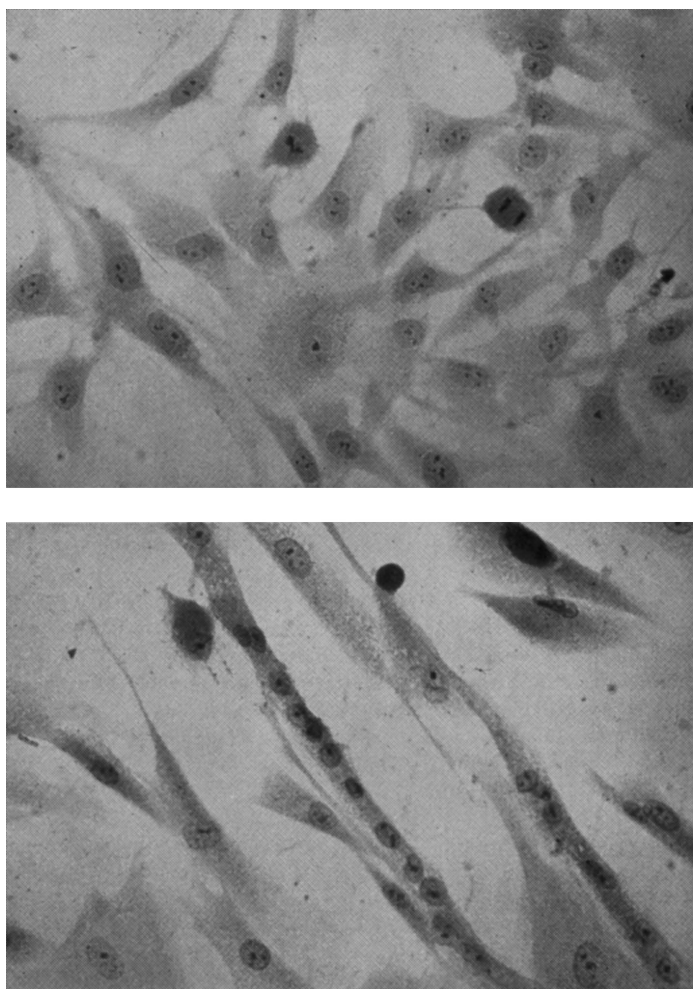


Fig. 7.6 Fetal muscle in tissue culture. Dividing uninucleate myoblasts (above). Fusion of myoblasts to form multinucleate myotubes (below).

With regard to the expression of dystrophin in cultured muscle, normally it is not demonstrable in undifferentiated myoblasts but appears only after myoblast fusion to form myotubes. We have shown that dystrophin is apparent already after 4 days in fusion media, although not all desmin-positive cells are also dystrophin-positive, confirming that desmin is a protein expressed much earlier during development. Dystrophin appears first in circumscribed areas in the sarcoplasm, but later, in more mature myotubes, it appears predominantly at the sarcolemma. Since these observations have been made with cultured aneural

muscle, and in the absence of 'trophic' nerve factors, the expression of dystrophin per se does not require innervation. As expected, in cultured Duchenne muscle, dystrophin is virtually absent, even in mature myotubes. Interestingly, reduced resistance to osmotic shock has been demonstrated in dystrophin-deficient myotubes, compared to normal ones, an indirect indication of increased sarcolemmal fragility in DMD muscle cells.

What light do these various studies throw on the pathogenesis of DMD? Perhaps one of the most interesting findings is an increase in intracellular calcium in aneurally cultured dystrophic muscle at a time when dystrophin would be expressed in normal tissue. However, the relationship between innervation, the expression of dystrophin, and subsequent intracellular events is complex. Muscle development, both *in vitro* and *in vivo*, involves the interplay of a number of myogenic factors, as well as innervation (see Fig. 7.7).

The interactions between these factors and dystrophin expression might be crucial in determining the full expression of dystrophy in cultured cells. As Valerie Askanas (personal communication) postulated:

... even though there is dystrophin deficiency in cultured Duchenne muscle, only some aspects of the abnormal phenotype, namely calcium accumulation (aneural cultures) and decreased CK-MM (innervated cultures), are expressed. It is possible that more advanced maturation of muscle is required for the full expression of an abnormal phenotype in Duchenne muscle in culture, which would be similar to the situation occurring *in vivo*...

Another important finding relates to the replicative ageing of myogenic cells (satellite cells) due to increased myofibre turnover. This has been correlated with progressive telomere reduction, which, by limiting the proliferative capacity of dystrophic human myoblasts, also limits their ability to be genetically

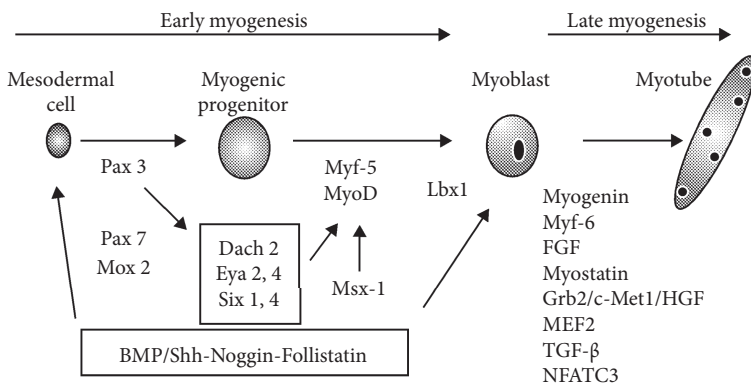


Fig. 7.7 The site of action of myogenic factors during muscle development.

modified and used for myoblast transplantation. Nevertheless, a more complex picture is emerging (Briggs and Morgan 2013). Indeed, not all satellite cells are functionally equivalent. Only a minority of satellite cells contributes to muscle regeneration. There are two populations of satellite cells. One population is responsible for myonuclei addition during growth and general muscle maintenance throughout life. The second population is formed by those satellite cells that are activated by severe muscle injury and survive transplantation. A sub-population of satellite cells has been shown to correspond to the 'stem' satellite cells that are capable of contributing to muscle regeneration and functionally reconstituting the satellite cell compartment. Regarding functionality, whilst it is clear that, in aged muscle, satellite cells have reduced telomerase length, indicating previous cycles of activation, transplantation studies have also suggested that they can efficiently contribute to regeneration in a normal muscle environment. This suggests that there are multiple reasons for the failure of regeneration potential in DMD, of which the abnormal 'niche' in which the satellite cells sit, perturbed by the remodelling of the extracellular matrix and the fibrotic process, plays a major role.

Cultured fibroblasts

The growth, behaviour, morphology, and biochemistry of skin fibroblasts in tissue culture from DMD patients have been studied extensively. Recently, the possibility that DMD fibroblasts might exert a paracrine effect by diffusible factors was suggested by experiments on Duchenne fibroblast/normal myoblast co-cultures. These studies also identified an altered expression of insulin growth factor-binding proteins (IGFBPs) mRNA, with the upregulation of IGFBP-5 and the downregulation of IGFBP-3 mRNA. The relevance of these findings is difficult to interpret, in view of the very low levels of dystrophin gene expression in fibroblasts.

Serum

Most studies have concentrated on the levels of various muscle enzymes in the serum of DMD patients. Elevated levels of most enzymes can be accounted for by their relative abundance in muscle tissue, as compared with serum, and their release from dystrophic muscle into the circulation. It has been suggested that enzymes may also be released from other organs, including the liver, but the evidence is not very convincing. Certainly, the characteristic liver enzymes γ -glutamyltransferase and sorbitol dehydrogenase are normal. It is interesting to note that a significant elevation of transaminases is

regularly found in DMD, and it is unfortunately not unusual, in this context, for several presymptomatic children with DMD or BMD to be extensively investigated for possible liver disease, before a neuromuscular disease was suspected. Some of these children undergo unnecessary invasive testing, such as a liver biopsy, before the muscle origin of these enzymes is suspected.

Novel technologies, such as mass spectrometry analysis of serum from DMD boys and in the *mdx* mouse model, are also leading to the precise quantification of proteins that are differentially regulated, compared to controls, such as factor XIIIa and serum matrix metalloproteinase-9 (MMP-9), that could represent potential biomarkers to monitor the disease severity and response to therapeutic intervention.

Urine

As in the case of serum, no abnormal metabolites have been detected in urine specifically in DMD. The changes in urinary composition that have been observed can all be explained on the basis of the release of various breakdown products from degenerating muscle into the circulation and then excretion in the urine. As we have seen, the urinary excretion of creatine is increased, whereas the excretion of creatinine is decreased, so that the ratio of creatine to creatinine in the urine is significantly increased.

Breakdown products excreted in increased amounts in the urine, when expressed in terms of creatinine, include carnitine, various amino acids, and 3-methylhistidine. The aminoaciduria in DMD is generalized, with no consistent pattern (plasma amino acid levels are normal). Frank myoglobinuria is not normally associated with DMD. However, we have reported a few instances of spontaneous myoglobinuria in toddlers with DMD.

None of these changes in urinary composition are specific to DMD but may be found in any neuromuscular disorder in which muscle breakdown occurs. The increased urinary excretion of dimethylarginines in muscular dystrophy, however, has a different origin. N^G , N^G -dimethylarginine is mainly located in cell nuclei as a component of non-histone nuclear protein, and its increased excretion reflects the myosin turnover in muscle regenerating from satellite cells. It is therefore an index of regenerative activity and could be a useful parameter for assessing the value of any proposed therapy.

Animal models

Various neuromuscular disorders have been described in many animals, including mink, sheep, duck, cow, dog, cat, hamster, and chicken, but only a few of these have turned out to be associated with secondary dystrophin deficiency.

These are the dystrophic mouse *mdx*, the dystrophic dog, and the dystrophic cat, and these will be discussed in the following sections. Of these, only the clinical picture of the dog model has significant resemblance to the human disease.

Muscular dystrophy in the mouse

In 1984, a spontaneous dystrophin mutant was identified in the C57BL/10 inbred strain, referred to as *mdx*. Points of similarity with DMD are the X-linked mode of inheritance, elevated serum levels of CK and PK, the primary involvement of skeletal muscle, and an absence of muscle dystrophin (human and mouse dystrophins are very similar). But there are significant differences from the human disease. The first and most important point is the lack of significant weakness in the *mdx* mice. Indeed, the *mdx* mouse strain was identified by the chance finding of elevated SCK, and not because of significant muscle weakness. Recent studies suggest, however, that aged *mdx* mice do show some degree of muscle weakness and might have a shorter lifespan. Also, the muscle histology is different, because, though muscle degeneration is evident early on, subsequently regeneration occurs, the regenerated fibres remaining centrally nucleated (see Fig. 7.8). Interestingly, the muscle degeneration starts at 2–3 weeks of age, affecting first the axial and proximal muscles and extending subsequently to the distal muscles. The regeneration is, however, very efficient, and the loss of muscle fibres and progressive fibrosis very limited. This is the main reason for affected mice remaining essentially normal, with no obvious weakness. The diaphragm is the only muscle to show progressive degeneration, although significant fibrosis has been demonstrated in various muscles in the old mice.

The molecular basis of the disorder in the *mdx* is a point mutation causing a stop codon in exon 23 of the dystrophin gene. Various chemical-induced

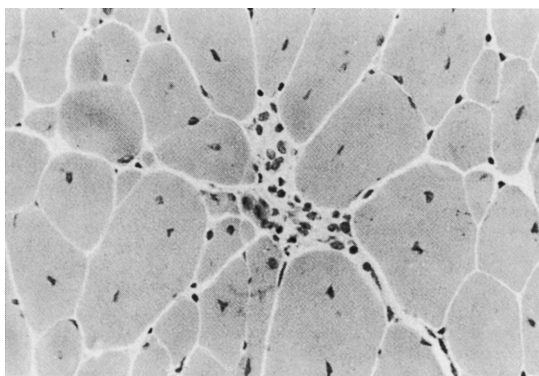


Fig. 7.8 Muscle histology in the mouse mutant *mdx*. Note the variation in fibre size and the preponderance of central nuclei (haematoxylin and eosin).

mutants have also been generated with point mutations in different parts of the gene, some of which affect also the 3' distal isoforms of dystrophin (for a review, see Noguchi and Hayashi 2001).

Although the *mdx* mouse provides a good model for some aspects of DMD, the most intriguing question it poses is why the disease is much milder than the human equivalent. An answer to this question could be of considerable significance in understanding the cause of progressive weakness in the human disease.

Muscular dystrophy in the cat

Dystrophin deficiency has also been described in male cats in which the histopathology is remarkable and shows muscle hypertrophy with progressive accumulation of calcium. Clinically, it seems unique in that diaphragmatic and glossal hypertrophy are predominant features, though the latter is not uncommon in DMD. The glossal hypertrophy can be severe, to the point that the affected cat can die because of malnutrition. Some affected cats can also develop severe muscle stiffness.

In the dystrophic cat, like the *mdx* mouse, but unlike the human disease and the dystrophic dog, there is no progressive loss of muscle fibres, no progressive fibrosis, and no progressive weakness, despite the deficiency of dystrophin. The molecular basis for the dystrophin deficiency in these cats is a deletion of the muscle promoter.

Muscular dystrophy in the dog

Breeds of dog with an X-linked muscular dystrophy, associated with a reduction or absence of dystrophin (which, in the dog, is identical to that in humans), are found in the golden retriever, the wire-haired fox terrier, the Rottweiler, the German short-haired pointer, and the King Charles spaniel. In many ways, these dystrophies are more comparable to DMD, because the muscle pathology is more or less identical to the human disease, and muscle weakness is progressive, with the later development of limb contractures. In the retriever, but not the fox terrier, occasional dystrophin-positive fibres occur, as in DMD.

The molecular basis for the disorder in the golden retriever model is a point mutation within the splice site of intron 6 of the dystrophin gene, causing a deletion of exon 7 from the transcript, with the loss of the open reading frame. So far, these breeds are the best animal models of the human disease and could provide excellent subjects for studying the effects of any therapeutic approaches to the disease. Both the Rottweiler and the German short-haired pointer have more severe clinical and pathological features than the golden retriever and also

suffer from dilated cardiomyopathy. The mutation in the King Charles spaniel is a splice site mutation that affects the splicing of exon 50, resulting in an out-of-frame deletion. This is relevant for therapeutic applications, as the technique of exon skipping, which will be discussed later on, is currently being pursued in the human, targeting an identical part of the human gene.

The study of the reasons for these species differences could be very revealing and help our understanding of the pathogenesis of the human disorder.

Summary and conclusions

Many biochemical abnormalities have been found in DMD. Abnormal findings are likely to be relevant to the pathogenesis, only when they relate specifically to DMD and occur in the **very early** stages of the disease process, before there is any significant muscle wasting and weakness.

Muscle tissue has been studied the most, and the observed biochemical changes are conveniently considered as being the consequence of three main processes. First, there are those changes that result from wasting and degeneration—these include the reduction in muscle myosin, carnitine, and most glycolytic enzymes. Second, there are changes attributable to the invasion of affected muscle by macrophages, lymphocytes, and fibroblasts, as well as to the necrosis of affected muscle fibres. These include the increase in enzymes present in fibroblasts and macrophages (such as NADP-linked dehydrogenases), proteases (cathepsins, lysosomal acid hydrolases, and calcium-activated proteases), and immune-related proteins and HLA antigens. Third, in many ways, dystrophic muscle resembles fetal muscle (histochemically and lipid, myosin, and isoenzyme patterns), for which the term ‘dedifferentiation’ has sometimes been used. The balance of evidence indicates that mitochondrial oxidation and sarcoplasmic reticulum and contractile protein functions are essentially normal in the **early stages** of the disease. However, a complex metabolic imbalance, secondary to the deficiency of a number of mitochondrial and glucose metabolism proteins, possibly linked to the absence of dystrophin, has been demonstrated in the later stage of the condition.

The results of studies on cultured dystrophic myoblasts have shown that there is a deficiency of dystrophin, but only some aspects of the abnormal phenotype are expressed by these cells in culture.

So far, the best animal models of DMD are the *mdx* mouse and certain breeds of the golden retriever. In all these models, the disease is X-linked and associated with a deficiency of muscle dystrophin. However, the disease is only progressive in the dog models, which could therefore provide the best animal models for therapeutic trials.

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Chapter 8

Genetics

Introduction to genetics

The familial nature of DMD was noted very early on by both Meryon and Gowers (see references in Chapter 2). In fact, as we have seen in Chapter 2, Gowers recognized that the disorder was limited to males and transmitted by healthy females, a mode of inheritance now recognized to be that of an X-linked recessive trait (see Fig. 8.1).

Mode of inheritance

Evidence of X-linked recessive inheritance includes not only the typical pedigree pattern, but also the fact that occasional female heterozygous carriers have had affected sons by different husbands. However, neither of these observations excludes the possibility that the disorder could be inherited as an autosomal dominant trait that is expressed only in males, so-called sex limitation. Two lines of evidence refute this. First, the disorder has been recorded in females with XO Turner's syndrome, XO mosaicism, or with a structurally abnormal X chromosome or an XY chromosome constitution. Second, statistical evidence indicates that the proportion of cases due to new mutations more closely resembles that expected for an X-linked recessive trait than for an autosomal dominant trait with male limitation. However, until the 1980s, the disease locus did not appear to be within measurable distance of other known X-linked loci. However, using DNA probes, as well as information generated by patients with cytogenetically identifiable deletions, the disease locus has now been shown to be located on the short arm of the X chromosome at position Xp21.

Penetrance

Complex segregation analysis in a large number of families with the disease indicates that mutations that completely disrupt the gene for DMD are always fully penetrant.

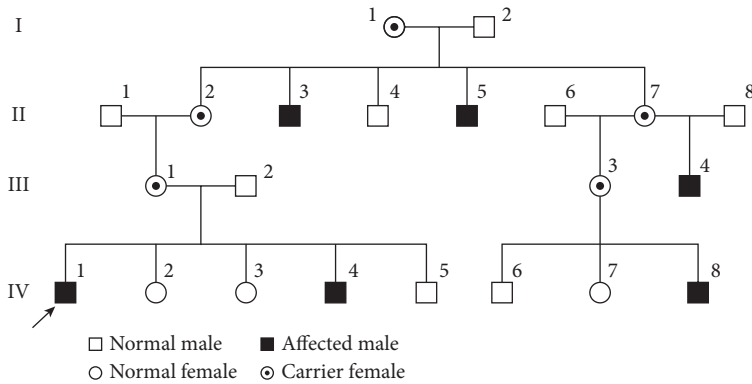


Fig. 8.1 Pedigree of a family with DMD.

Incidence

In order to determine the frequency of the responsible gene and its rate of mutation, it is necessary to determine the population frequency of the disorder. This information is also essential in order to determine if various preventive measures are being effective and to help in the planning of adequate resources and welfare services for affected families.

Incidence refers to the number of **new** cases occurring per unit of population. Prevalence, on the other hand, refers to **all cases present in the population**, either within a given period (so-called period prevalence rate) or at a particular point in time (so-called point prevalence rate) per unit of population at risk at that time. In the case of DMD, prevalence, particularly after early childhood, would be less than the true incidence at birth because of increasing mortality.

Since the disorder is not clinically recognizable at birth, the birth incidence is usually derived from knowing the number of normal boys born in the same years that affected boys were born. However, a small proportion of normal boys may die by the age at which affected boys are diagnosed. It has therefore been argued that the incidence should perhaps be related not to the number of normal live births, but rather to the number of normal children who survive to the age at which affected boys are diagnosed. But some affected boys may also die, before clinical manifestations become evident. The best compromise seems to be to calculate the incidence from the assumed frequency at birth as a proportion of all births.

Incidence may also be derived from prevalence by taking into account the probability of ascertaining affected individuals in the population and the probability of an individual developing the disease by a given age.

Some significant variations between the estimates of incidence and prevalence in various countries have been reported, including differences even within a single country such as the UK or Italy. The highest incidences between the 1960s and 1980s were reported in Emilia (north-east Italy, 390×10^{-6}), north-east England regions (311×10^{-6}), and the Erfurt region in Germany (307×10^{-6}), whilst the lowest incidences were recorded in Poland (140×10^{-6}) and Friuli (Italy, 97×10^{-6}). Regarding population prevalence figures, in the same period of time, they ranged upwards, from 17.2×10^{-6} in Kumamoto (Japan) to 69.3×10^{-6} in Emilia (Italy). A summary of the incidence and prevalence data published from all over the world is given in Table 8.1.

Despite increasing awareness of the disease, there has been little decrease in reported incidences. It could be that any inflation by the inclusion of other forms of dystrophy in the past has been balanced in more recent studies by improved ascertainment of true cases. The overall incidence, based on over 10 million live male births, is $206 \pm 4 \times 10^{-6}$, or one case in 4854. In several studies performed up to the early 1990s, the birth incidence approaches 300×10^{-6} , or roughly 1 in 3500 male births (Emery 1991).

The birth incidence of the disease can also be determined by screening for a raised SCK level in the neonatal period (see Chapter 11) and subsequently confirming the diagnosis by muscle histology, followed by dystrophin studies and gene deletion analysis. These screening programmes first began in the 1970s, with subsequent reports from New Zealand, Scotland, France, and Germany. Since then, there have been a number of technological improvements, and results from several extensive studies are summarized in Table 8.2 where the overall incidence is given as $199 \pm 14 \times 10^{-6}$, or roughly 1 in 5000.

Nevertheless, more recent studies, which include >20 years of newborn screening programme in Wales, all appear to point to an average DMD incidence of ~1:5000 newborn males (Mendell *et al.* 2012; Moat *et al.* 2013).

These studies clearly show that neonatal screening for DMD is feasible and, coupled with molecular genetic testing and muscle biopsy, is effective in

Table 8.1 DMD: prevalence in the population and incidence in live male births*

Number affected	Total population	Prevalence (mean \pm SE) $\times 10^{-6}$	Number affected	Total LMB	Incidence (mean \pm SE) $\times 10^{-6}$
1956	46 282 453	42 ± 1	2111	10 236 248	206 ± 4

LMB, live male births; SE, standard error.

* These data are based on some 50 reported studies, covering the period 1939–90. Detailed references can be found in the second edition of this book.

Table 8.2 Results of various neonatal screening programmes for DMD, reported at the 14th ENMC Sponsored Workshop 'Screening for Muscular Dystrophy', 5–6 March 1992, Baarn, The Netherlands (Chairman: Professor G. -J. van Ommen)

Centre	Start of programme	Age tested	Total screened ($\times 10^3$)	False positive (%)	Proven DMD
Manitoba, Canada	1986	1–5 days	54	0.10	10
Lyon, France	1975	5 days	328	0.19	60
		3 days		0.40	
Philadelphia, US	1986	1–4 days	49	0.30	10
Antwerp, Belgium	1975	5–7 days	150	0.02	25
Cardiff, Wales	1990	6–7 days	24	0.02	9
Germany*	1997	4 weeks–6 months	358	0.02	78
<i>Total</i>			963		192
<i>($I = 199 \pm 14 \times 10^{-6}$)</i>					

* Voluntary screening.

Source: data from 14th ENMC Sponsored Workshop 'Screening for Muscular Dystrophy', 5–6 March 1992, Baarn, The Netherlands.

diagnosing affected children early. Currently, neonatal screening programmes are under way in several countries. There are, however, both practical and ethical issues that have so far prohibited a more widespread use of this early diagnostic technique. These will be discussed in detail in Chapter 11.

Changes in incidence in recent years

In recent years, the apparent reduction in the incidence of DMD might be expected as a result of several factors. Beginning in the 1950s, there was an increase in interest in genetic counselling, and the mid 1960s saw the advent of carrier detection tests. Later, prenatal fetal sexing became possible so that a mother who was at high risk of having an affected son could request selective abortion of any male fetus in any subsequent pregnancy. Finally, in the recent past, prenatal diagnosis of affected male fetuses has become possible, using molecular genetic testing. Genetic counselling and carrier detection studies in affected families might be predicted to have resulted in some reduction in incidence. In a well-designed and careful study in western Japan, a decrease in incidence, from 223×10^{-6} for the period 1956–60 to 145×10^{-6} for the period 1976–80, was

Table 8.3 Results of more recent neonatal blood screening programmes for DMD, reported at the 195th EMNC Sponsored Workshop, December 2012 (organized by Ellis J., Vroo E., and Muntoni, F.)

Year	Location	NBS/DMD	Incidence
1979	New Zealand	10 000/2	1:5000
1982	Edinburgh, UK	2336/0	0
1986	West Germany	358 000/78	1:4589
1988	Manitoba, Canada	54 000/10	1:5400
1989	Lyon, France	37 312/10	1:5330
1991	WPA, US	49 000/10	1:4900
1998	Cyprus	30 219/5	1:6002
2006	Antwerp, Belgium	281 214/51	1:5500
2011	Wales, UK	335 045/73	1:5266
2012	Ohio, US	3774/6	1:6291

Source: data courtesy of Jerry Mendell.

noted and attributed to the effects of genetic counselling (Takeshita *et al.* 1977, 1987). These data appear to be substantiated by more recent reports of Moat *et al.* (2013) and Mendell *et al.* (2012) (see Table 8.2 and Table 8.3).

Mutation rate

The rate at which the gene causing DMD mutates may be estimated either indirectly or directly.

Indirect estimation of mutation rate

For any X-linked recessive disorder, if the reproductive fitness of affected individuals is *f* and the incidence of the disease is *I*, then the mutation rate is:

$$\text{Mutation rate} = 1/3I(1 - f)$$

However, in DMD, the biological fitness is zero, because affected boys do not procreate. Therefore, the mutation rate is given by 1/3.

If the incidence of the disorder is assumed to be around 200–300 × 10⁻⁶, then the mutation rate is around 70–100 × 10⁻⁶ genes per generation.

Direct estimation of mutation rate

In the direct method, an attempt is made to estimate the actual number of new mutations among isolated cases. If *a* of *b* known female carriers have an

abnormal SCK level, then the detection rate of carriers is a/b . If n isolated cases are born in a given period among N males born in the same period, and if c is the number of mothers of these n males who have an **abnormal** SCK level, then, among these isolated cases (subject to sampling error), the number of **new** mutations will be:

$$n - (bc / a)$$

And therefore the mutation rate is:

$$n - (bc / a) / N$$

In one study (Gardner-Medwin 1970), 22 of 35 known carriers had raised SCK levels. Of 56 mothers of isolated cases, 15 had raised levels. Thus, the proportion of new mutations (mothers are non-carriers) among isolated cases was:

$$[56 - 15(35 / 22)] / 56 = 0.574$$

Over a 9-year period (1952–60), 43 isolated cases were born, and therefore the number of new mutations was:

$$43 \times 0.574 = 24.682$$

The total number of males born in this period who survived to age 5 (by which time almost all cases of DMD are diagnosed) was 236 200. Thus, the mutation rate was:

$$24.682 / 236\,200 = 105 \times 10^{-6}$$

The estimates of the mutation rate by both these methods are considerably greater than values obtained for other X-linked disorders. For comparison, some representative values for various genetic disorders are given in Table 8.4. The only disorder with a comparable mutation rate is neurofibromatosis, but this is now known not to be a single genetic disease. The very high mutation rate in DMD is probably a reflection of the enormous size of the dystrophin gene, which therefore provides a bigger target for mutagenic agents.

Parental age and mutation rate

In X-linked recessive disorders, possible effects of maternal or paternal age on mutation rates can be assessed separately by considering, respectively, the maternal age in the case of mutant males, and the maternal grandfather's age in the case of mutant heterozygous mothers. The latter are mothers with no affected relatives other than sons (or who have only one affected son, but a significantly elevated SCK level) where the new mutation can have occurred in the X chromosome she inherited from her father.

Table 8.4 Average estimates of mutation rates for various genetic disorders (data from various sources)

Disorder	Mutation rate ($\times 10^{-6}$)
Autosomal dominants	
Achondroplasia	6–13
Retinoblastoma	5–12
Tuberous sclerosis	6–10.5
Polypsis coli	13
Neurofibromatosis	44–100
Huntington's chorea	5
Myotonic dystrophy	8–11
Autosomal recessives	
Albinism	28
Total colour blindness	28
Phenylketonuria	25
X-linked recessives	
Haemophilia A	32–57
Haemophilia B	2–3

None of the studies that have considered this problem have found any significant increase in the age of mothers of presumed new mutants. With regard to presumed mutant heterozygous mothers, the mean ages of both grandparents at the birth of these mothers can be compared with the mean ages of the mothers' spouses' parents at the birth of their spouse. In several studies, the mean maternal grandfather's age was greater than the mean paternal grandfather's age (see Table 8.5), but the differences were not statistically significant.

If there is any paternal age effect on the mutation rate in this disorder, it would seem to be negligible. The results of these studies do not support the idea that the mutation rate in males is significantly different from that in females.

Sex difference in mutation rate

It can be shown that, if a mother has an affected son but no one else in the family is affected, then the probability of her being a carrier is:

$$(\mu + \nu) / (2\mu + \nu)$$

(where μ is the mutation rate in female germ cells, and ν is the mutation rate in male germ cells)

Table 8.5 Ages (years) of grandparents of cases of DMD where the mother is presumed to be a mutant heterozygote

Maternal grandparent			Paternal grandparent			Difference	Year of study
Number	Mean age	SD	Number	Mean age	SD		
Grandfathers							
26	34.3	6.14	22	30.9	6.42	+3.4	1977
15	35.1	7.03	15	31.4	6.43	+3.7	1980
82	34.1	7.33	81	33.9	7.56	+0.5	1982
Grandmothers							
26	30.5	4.87	24	27.8	5.47	+2.7	1977
14	30.4	6.63	16	27.4	5.48	+3.0	1980
82	30.3	7.06	82	29.0	6.07	+1.3	1982

The probability that she is **not** a carrier and that the son is therefore the result of a new mutation is:

$$1 - [(\mu + \nu) / (2\mu + \nu)] = \mu / (2\mu + \nu)$$

This represents the proportion of new mutants, often designated as ' x '. If the mutation rates are the same in both males and females, then x is one-third; if mutations occurred more frequently in the male, then x approaches zero, and, if mutations occurred more frequently in the female, then x approaches one-half.

This is not just of academic importance, because, if mutations were found to occur exclusively in the male, then all mothers with an affected son would be carriers, and this would be important in genetic counselling.

Several different methods have been devised for estimating x . Essentially, there are three approaches:

- ♦ analysis of sibships;
- ♦ sex ratio of unaffected sibs;
- ♦ methods based on the results of carrier detection tests.

With regard to the sex ratio method, this is independent of ascertainment and is based on the assumption that, among the offspring of a **carrier**, affected boys, unaffected boys, and girls will, on average, occur in the ratio 1:1:2. Therefore, the sex ratio (M:F) among unaffected sibs will be 1:2 (or 1:1.89 if corrected for the deviation of the sex ratio from 1). However, among the sibs of **new mutants**, the sex ratio will be 1:1 (or 1:0.94 if corrected). The proportion of new mutants among isolated cases can be estimated by determining the sex ratio among the sibs of isolated cases.

Finally, with regard to carrier detection methods, these have usually been based on comparing the proportion of abnormal test results (the most reliable in the past being the SCK level) in known carriers with mothers of isolated cases. Thus, if i is the proportion of mothers of isolated cases with an elevated SCK level, d is the proportion of known carriers with an elevated SCK level, and P_i is the proportion of isolated cases among all cases, assuming complete ascertainment in a given population, then:

$$x = (1 - i / d)P_i$$

The results of some 20 studies of the problem indicate that x would accumulate a value of 0.33, which assumes that mutations are equal in males and females.

The possibility of **germline mosaicism** must also be considered as a possible explanation for lower values of x . In an extensive study of a pooled sample of 1885 sibships from seven different countries, the proportion of sporadic cases was estimated to be 0.229 ± 0.026 (Barbujani *et al.* 1990). From these data, it can be calculated that the upper 95% confidence limit for x is 0.280, and therefore the proportion due to mosaicism in apparently non-carrier mothers would be expected to be at least 16%. This figure is close to that obtained from molecular studies (see Chapter 11).

The increasing election by mothers, after the birth of an affected son, of family limitation and selective abortion in future pregnancies may well invalidate the assumptions underlying these various approaches. These practices will lead to an increasing proportion of isolated cases and a decreasing sex ratio (M:F) in subsequent sibs.

Finally, a collaborative international investigation of the problem using DNA haplotypes, in order to identify the origin of mutations within families, indicates that the mutation rates in males and females are roughly equal (Muller *et al.* 1992).

So far, it has been assumed that all cases result from a mutation in the germ cells. But if the male twins discordant for DMD, reported by de Grouchy in 1963, were, in fact, identical, as the authors stated, then this raises the possibility that mutations may also occur after conception. Post-zygotic mutation would also account for a reported pair of MZ twins being discordant, for example, for tuberous sclerosis. There could be other examples.

Summary and conclusions

Evidence from various sources, including pedigree studies, affected girls with X chromosome abnormalities, and statistical methods has shown that DMD is

inherited as an X-linked recessive trait. The mutant gene is always fully penetrant, and a subclinical, as opposed to a preclinical, form of the disease does not exist. Estimates of the incidence of the disorder, based on population surveys, vary but are probably around $200\text{--}300 \times 10^{-6}$. This puts the mutation rate at around $70\text{--}100 \times 10^{-6}$ genes per generation, which is considerably greater than in any other disorder and reflects the enormous size of the dystrophin gene, which is therefore a greater target for mutagenic agents. A possible difference in mutation rates in male and female germ cells has been studied by considering maternal and grandpaternal age effects, as well as the proportion of isolated cases that could be due to new mutations. The results of all these investigations indicate that the mutation rates in male and female germ cells do not differ significantly, and around one-third of isolated cases of the disease are due to new mutations.

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Molecular pathology

Introduction to molecular pathology

In order to unravel the molecular pathology of a disease, where the basic biochemical defect is unknown, various approaches are possible. These have been well exemplified in the case of the discovery of the DMD gene which represented a technological cornerstone for the 80s. First, the gene has to be localized to a specific chromosome, and then to a particular site on the chromosome. Second, armed with such information, DNA markers can be selected that are located in this particular region of the chromosome, and, if they prove to be closely linked to the disease locus, they can be used for carrier detection and prenatal diagnosis. Third, it may be possible, using molecular techniques, to 'walk the genome' from the DNA markers toward the mutant gene, so as to eventually include the gene itself. As will be seen, however, there have been several other strategies pursued to isolate the Duchenne locus. Fourth, having isolated the gene, or at least part of it, this can then be used as a 'gene-specific' probe for direct prenatal diagnosis and carrier detection. Finally, having isolated the gene, it is then possible, by DNA amplification and sequencing, to define the nature of the molecular defect and its product. Each of these steps will be described in relation to DMD, although much of the detail is really outside the scope of this book and is furnished in the relevant bibliography.

Localization of the Duchenne gene

An early clue as to the specific location of the Duchenne locus came from the study of rare cases of a Duchenne-like disorder in girls with X/autosome translocations (Ray *et al.* 1985). The first cases were described in the late 1970s. Since then, some 20 or so similar cases have been reported. Full details with references are given in the second edition of this book. In each case, the findings have been consistent with the diagnosis of a muscular dystrophy, with grossly elevated SCK levels and myopathic changes on EMG and muscle pathology. Most have been clinically similar to DMD, although, in some cases, the disorder seemed less severe and perhaps more like BMD. Some girls have been mentally retarded. All, however, have had a reciprocal translocation between an autosome and the X chromosome, and, since these are balanced translocations, with

no apparent loss of chromosomal material, these girls might have been expected to be normal. In such X/autosome translocations, however, it is the normal X that tends to be preferentially inactivated, with the result that genes on the derived (der) X are expressed. It could therefore be that the mothers of these girls were heterozygous carriers and that the maternal X chromosome carrying the mutant gene was involved in the translocation. However, this is unlikely, because in only two instances has there been any suggestion that the mother might be a carrier and that the translocation chromosome was, in fact, of paternal origin. Also, the parents of these girls have had normal chromosomes, and we are therefore left to conclude that both the translocation as well as the disease must have arisen *de novo* in the affected girls.

Since different autosomes were involved in the various translocations, but the breakpoint on the X chromosome was **always** in the region of Xp21, the most likely explanation is that the translocation, in some way, disrupted the normal gene at Xp21, which then resulted in the disease. The mutant gene was therefore presumed to be located at this point on the X chromosome (see Fig. 9.1). There is considerable variation in clinical severity among girls with X/autosome translocations and a Duchenne-like disorder. It seems possible that the phenotype may well depend on the proportion of cells in which the der X is active. The milder the phenotype, the lower the proportion of cells in which the active X chromosome is the der X, and therefore the greater the proportion of cells in which the active X chromosome is the normal X. In fact, the published data indicate that the proportion of cells in which the active X is the der X was somewhat lower in cases considered to be mild.

X/autosome translocations involving other breakpoints on the X chromosome have also been described. In these cases as well, the expression of various X-linked disorders could be attributed to the disruption of the normal alleles at the respective loci (see Table 9.1).

Whilst studies of X/autosome translocations in females with muscular dystrophy were in progress, a unique case was described that further confirmed the location of the Duchenne locus. This concerned a boy with DMD and some degree of mental retardation, who also exhibited chronic granulomatous disease, McLeod syndrome (reduced antigenicity of the red cell Kell blood group), and a form of retinitis pigmentosa. High-resolution chromosome banding studies revealed that the band Xp21 appeared to be slightly reduced in size, and molecular studies confirmed that the affected boy had a small interstitial deletion in this region. This deletion presumably removed DNA sequences at the DMD locus, as well as at other adjacent loci, thus producing the clinical phenotypes. This case further supported the idea that the Duchenne locus was at Xp21. It also indicated that the loci for these different disorders are clustered together. In fact,

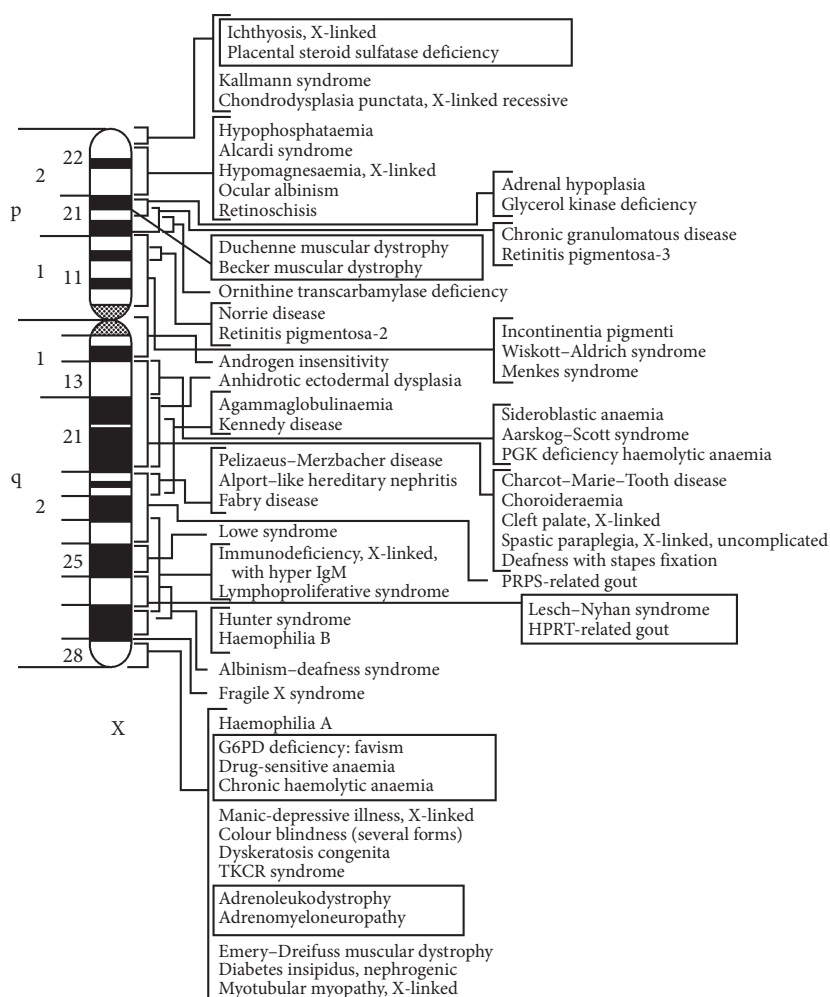


Fig. 9.1 Gene map of the X chromosome and its banding pattern.

Reproduced from McKusick, Victor A., MD, Clair A. Francomano, MD, and Stylianos E. Antonarakis, MD, DSc, *Mendelian Inheritance in Man*, tenth edition, Volume 1. pp. cci, Figure B2d, Copyright © 1966, 1968, 1971, 1975, 1983, 1986, 1988, 1990, 1992 Johns Hopkins University Press. Reprinted with permission of Johns Hopkins University Press.

various associations of these different disorders have been reported in males: chronic granulomatous disease with McLeod syndrome; McLeod syndrome with raised SCK levels and a subclinical myopathy; chronic granulomatous disease with McLeod syndrome and raised SCK levels; chronic granulomatous disease with DMD; chronic granulomatous disease, McLeod syndrome, DMD, and a form of retinitis pigmentosa. The gene for ornithine transcarbamylase (OTC)

Table 9.1 Expression of X-linked disorders in females with various X/autosome translocations

Disorder	X breakpoint
DMD and BMD	Xp21
Anhidrotic ectodermal dysplasia	Xq12
Aicardi's syndrome	Xp22
Hunter's syndrome	Xq28
Aarskog syndrome	Xq13
Incontinentia pigmenti	Xp11
Lowe's syndrome	Xq25
Menkes syndrome	Xq13

is also localized to Xp21 and has been shown to be relatively closely linked to the Duchenne locus. A small, but visible, deletion, associated with OTC and glycerol kinase deficiencies and X-linked adrenal hypoplasia, has been described. Also, a deletion in the same region of the X chromosome has been found in boys with myopathy, glycerol kinase deficiency, and adrenal insufficiency. This triad has also been reported when there is no visible deletion, but a deletion may then be detected using an appropriate DNA probe. The triad may also be associated with other abnormalities. Glycerol kinase deficiency and adrenal hypoplasia have been recorded in different male members of a particular family, but in the absence of a myopathy, and the glycerol kinase locus is clearly distinct from the Duchenne locus. Aland Island eye disease can also occur in association with DMD, glycerol kinase deficiency, and congenital adrenal hypoplasia. We know of another instance with a large deletion in three siblings with BMD, glycerol kinase deficiency, adrenal hypoplasia, undescended testes, and mental retardation, which led to the identification of a novel gene (interleukin-1 receptor-related protein, IL1RAPL1). Interestingly, in these brothers, the dystrophin gene was fused tail-to-tail with the IL1RAPL1 gene. Mutations in this gene have been also reported in non-specific X-linked mental retardation.

On the basis of these reports of overlapping phenotypes, it is possible to order the responsible gene loci (see Fig. 9.2). Because of the involvement of contiguous genes, these have been referred to as contiguous gene syndromes. This arrangement of the disease gene loci around the Duchenne locus has now been confirmed by detailed gene mapping studies.

Cases of DMD with no other associated abnormalities, but with a microscopically evident interstitial deletion at Xp21, are very much the exception. Such cases have been described by several authors, where the deletion was large

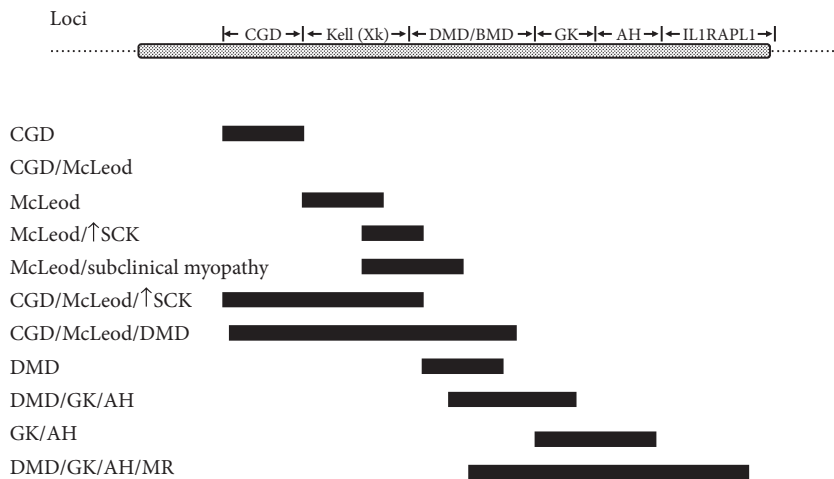


Fig. 9.2 Diagrammatic representation of contiguous gene syndromes involving chronic granulomatous disease (CGD), McLeod syndrome (Kell), DMD, BMD, glycerol kinase deficiency (GK), adrenal hypoplasia (AH), and mental retardation secondary to mutations in the interleukin receptor 1-like gene (IL1RAPL1).

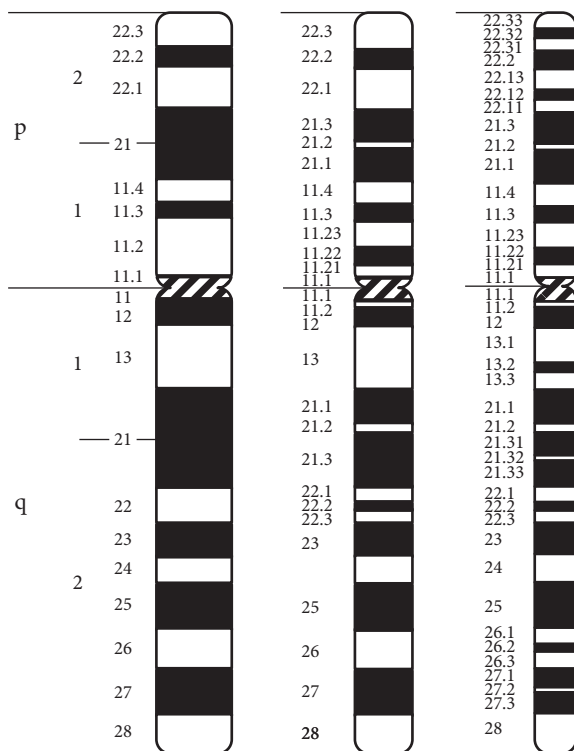


Fig. 9.3 Diagrammatic representation of the high-resolution banding patterns that can be delineated on the X chromosome by various techniques.

enough to be visible but was presumably not sufficiently extensive to include any adjacent gene loci. In the vast majority of cases of DMD, however, no deletion or any other alteration is microscopically evident in the region of Xp21, even with high-resolution banding. Such techniques reveal that the band Xp21 can be subdivided into three regions (see Fig. 9.3), and, when applied to lymphoblastoid cell lines from females with a Duchenne-like disorder and an X/autosome translocation, the breakpoint has been found to be in sub-band Xp212 or in Xp211. It can be calculated that the DNA represented in the sub-bands Xp211, Xp212, and Xp213 is around 5000, 2000, and 4000 kb, respectively (1 kb = 1000 base pairs (bp) of DNA). From these cytogenetic studies, the chromosomal region involved in some way with DMD/BMD would therefore span >1000 kb of DNA. In fact, these results have been confirmed by later genomic studies that have shown that the dystrophin locus spans ~3 mb, constituting roughly 3% of chromosome X.

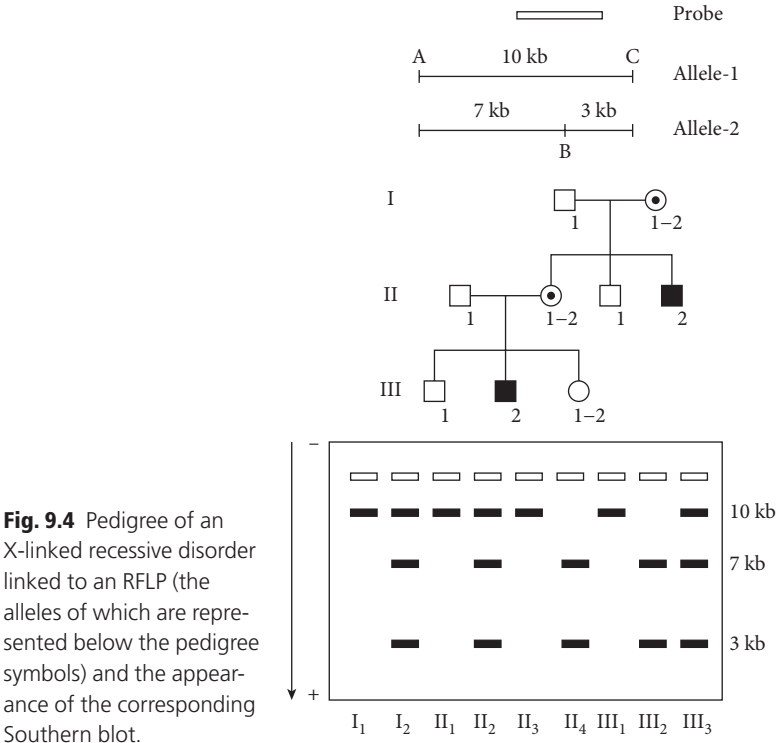
Linked DNA markers

Between genes, there are large stretches of DNA, the functions of which are still largely unknown. Within this DNA are variations in nucleotide sequences or single nucleotide variations that have no apparent phenotypic effects on the host organism and are inherited in a Mendelian fashion. These changes in base sequence, which occur about once in every 100 bp, can be identified, because they can alter the DNA site normally cleaved by a particular restriction enzyme, since these enzymes cleave DNA at sequence-specific sites. Thus, a change in base sequence in a segment of DNA will, with a particular restriction enzyme, result in different-sized fragments in different individuals. These genotypic changes can be recognized in different ways. Until the early 1990s, they could only be studied by the different mobilities of the restriction fragments on gel electrophoresis. The fragments were identified by using an appropriate 'probe'—a labelled DNA fragment that will hybridize with, and thereby detect, complementary sequences among the DNA fragments produced by a restriction enzyme (Southern blot). These variations in nucleotide sequences are referred to as RFLPs. Their interest lies in the fact that the demonstration of linkage between an RFLP and the locus for a particular genetic disease can be useful for carrier detection and prenatal diagnosis. Also, if the chromosomal site of an RFLP were already known, it could furnish information on the site of a disease locus to which it proved to be closely linked.

Subsequently, the discovery of polymorphic microsatellite markers and of single nucleotide polymorphisms (SNPs) has greatly enhanced the possibility of detecting variations between individuals. The former typically are represented

by stretches of di- or trinucleotides that are often highly polymorphic in the general population. They represent an advantage, compared to RFLPs, because they can be easily assessed following a simple PCR assay, often have a significantly higher heterozygosity index, and are randomly located in the genome at intervals that, on average, are <1 map unit or centimorgan (cM; 1 cM is roughly equal to 1000 kb or 106 bp) apart. This means that the majority of genes will have several polymorphic microsatellite markers located in close proximity or within the introns of the genes themselves. This is the case also of the dystrophin gene, in which a number of intragenic polymorphic microsatellite markers have been identified. They therefore represent very powerful tools for studying the linkage of a particular locus in family studies.

A hypothetical example of the co-inheritance of an RFLP and an X-linked recessive disorder is given in Fig. 9.4. Here, it is assumed there is a polymorphism at restriction site B—the absence of the site is called allele-1, and the presence of the site is allele-2. When the restriction enzyme cuts the DNA in one chromosome at sites A and C, it generates a single fragment of size 10 kb, which corresponds to allele-1. If the enzyme cuts the DNA not only at sites



A and C, but also at B, two fragments will now be generated of sizes 7 and 3 kb, respectively, which correspond to allele-2. Polymorphic genotypes can therefore be deduced from the pattern of bands on an electrophoretic gel. In this example, the grandmother (I_2) and mother (II_2) are both heterozygous for the RFLP, and both carry the X-linked recessive disorder. Since both affected males have allele-2, it would appear that allele-2 and the disorder are co-inherited in this family. That is, they are both on the same X chromosome.

By studying individuals in a family in which an RFLP and an X-linked recessive disorder are inherited, it is possible to estimate how frequently crossing-over occurs between them. Thus, in this example, if the unaffected son III_1 had also inherited allele-2, there would appear to have been at least one cross-over (or recombinant) out of four meioses. By studying a number of informative families in this way, the frequency of recombination can be determined. Recombination frequency, usually designated as θ , is related to the distance between the gene loci concerned—1% recombination being equivalent to a distance of 1 cM. When loci are some distance apart, then the error rate in diagnosis will be equal to θ at each meiosis.

With regard to linkage with DMD, the first step was to isolate a relevant DNA sequence. In 1981, Davies and her colleagues isolated the first DNA sequences from cloned fragments derived from the human X chromosome, the so-called X genomic library. The location of these cloned sequences was then determined by various methods. These included studying somatic cell hybrids with a full complement of Chinese hamster or mouse chromosomes and different extents of the human X chromosome. Another method of localizing a DNA sequence was by *in situ* hybridization whereby the sequence was labelled and hybridized directly to a metaphase chromosome preparation. One of the cloned sequences, designated RC8, turned out to be located on the short arm of the X chromosome. Using this as a probe, it detected a polymorphism with the restriction enzyme *TaqI*, and, by studying several families, the polymorphism detected by RC8 (now called DXS9) proved to be linked to DMD. Shortly afterwards, a different probe (L1 0.28) detected another polymorphism (DXS7) on the opposite side of Xp21, and it was found that the Duchenne locus lay between the two. Both markers eventually turned out to be about 15 cM on either side (referred to as 'flanking' or 'bridging') of the Duchenne locus. Not only was this a landmark in the history of the disease, but it was the first disorder shown to be linked to an RFLP (Davies *et al.* 1983).

Around the same time, others showed that BMD was linked to DXS7 (L1.28). Subsequently, it was also shown to be linked to DXS9 (RC8) and to other DNA markers at roughly similar genetic distances to those in DMD. These findings therefore indicated that DMD and BMD could either be allelic

or that the two loci could be very close together in the same region of the X chromosome. We now know that they are, in fact, allelic. Interestingly, Emery–Dreifuss muscular dystrophy was shown to be linked to colour blindness and to DNA markers located at Xq28, which indicated that the responsible gene mutation was not allelic with DMD and BMD. We now know that the gene responsible for X-linked Emery–Dreifuss muscular dystrophy is the *STA* gene, located at Xq28.

The identification of dystrophin intragenic polymorphic microsatellite markers has recently improved the study of carriers in families with DMD or BMD.

Isolation of the Duchenne gene

The most obvious approach to eventually isolating the Duchenne gene itself would a priori have been to ‘walk the genome’ from a closely linked probe toward the gene, so as to eventually include it. This could be done by studying overlapping clones of DNA sequences. There are several reasons, however, why this approach proved difficult. Even a very closely linked marker only 1 cM from the disease locus is a million bp away, and it would be extremely difficult to know if one were moving closer or further away by this strategy. Other methods seemed more likely to succeed.

One approach, pursued by Worton and his colleagues in Toronto, Canada, was to isolate the junctional region in an X/autosome translocation associated with a Duchenne-like disorder in a female. They chose a translocation involving chromosome 21, which they showed split the block of genes encoding the ribosomal RNA on the short arm of chromosome 21 (Worton *et al.* 1984). They then used ribosomal RNA probes to identify the junctional fragment in clones derived from the region of the translocation site. The region spanning the translocation breakpoint, and which presumably contained at least part of the Duchenne locus, was then cloned. A sequence derived from the clone was found to detect an RFLP that was very closely linked to DMD. This probe (referred to as XJ probe) failed to hybridize with DNA from the occasional patient with DMD, indicating that, in these boys, there is a deletion of the region complementary to the probe.

Kunkel and colleagues in Boston approached the problem in a different and particularly ingenious way. They extracted DNA from a patient with a large deletion spanning the locus. The DNA was then sheared by sonication which produces DNA fragments with irregular ends. DNA from a 49, XXXXY lymphoid cell line was cleaved with the restriction enzyme *Mbo*I. The two sets of fragments were then mixed and heated in order to disassociate the DNA

strands. These were then allowed to reassociate in the presence of phenol (the so-called phenol-enhanced reassociation technique, PERT). Under appropriate conditions, and with the patient's DNA in excess, most of the reassociated molecules will have sheared ends, and a few will be hybrid molecules, with one sheared end and one *MboI* 'sticky end'. However, those sequences in the control not represented in the patient's DNA (where they are deleted) will **not** hybridize with the patient's DNA. Perforce, they will only hybridize between themselves and therefore consist of perfectly reassociated molecules with two *MboI* ends. Only the last can be ligated into an appropriately cleaved plasmid and be cloned (Kunkel *et al.* 1985). In this way, a library of cloned sequences (referred to as PERT probes), corresponding to the portion of DNA deleted in the affected boy, was produced. These have detected several RFLPs closely linked to the Duchenne locus, and, like Worton's probes, they also detected small deletions in a proportion of affected boys (Monaco *et al.* 1985), which are of different lengths in different families (Kunkel *et al.* 1986).

It should be noted that the probes isolated by Worton (XJ) and Kunkel (PERT) have shown recombination with DMD ($\theta \cong 0.05$), thus further indicating that the Duchenne locus is very large. In fact, recombination between markers at the two extremes of the locus is at least 10%. But recombination may not be uniform throughout the locus. A hot spot, for example, is centred around DXS 164 (PERT 87), located in intron 11.

Deletions

The Xp21 locus has been cloned, and, using cDNA probes derived in this way, ~70% of cases of DMD and over 80% of cases of BMD have been found to have gene deletions. These findings have two important implications. First, if the deletion involves a significant part of the gene, then it might be expected that the affected individual could mount an immune response, if exposed to the missing gene product, and this could make replacement therapy difficult. Second, it is possible to track down the origin of the deletions and identify the occurrence of *de novo* events. This is illustrated in Fig. 9.5, based on two families. Using a particular probe (PERT 87–8), an RFLP was detected with the enzyme *BstXI*, reflected in two DNA fragments of sizes 4.4 and 2.2 kb (say, allele-1 and allele-2, respectively).

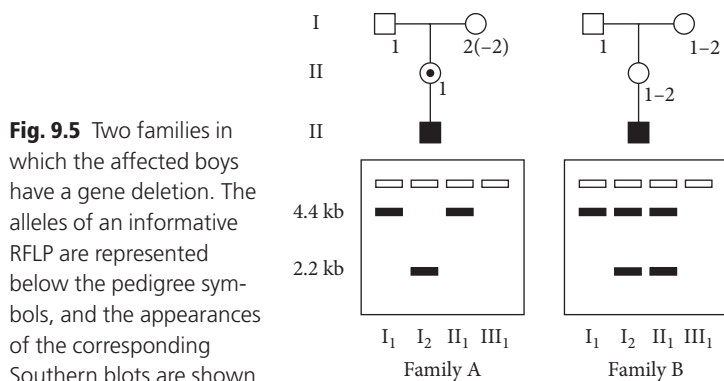
- ◆ In family A, the grandmother (I_2) is either homozygous for allele-2 or hemizygous, because one allele has been deleted. Her daughter (II_1) has no allele-2, which she should have inherited from her mother. She is therefore heterozygous for the deletion that is evident in her affected son (III_1) who is therefore **not** a new mutation.

- ◆ In family B, both the grandmother and mother are heterozygous for the RFLP, and neither allele is deleted. However, one of the alleles is deleted in the affected son who must therefore be a **new** mutation.

Though up to 70% of cases of DMD have a deletion, a proportion have a gene duplication, which probably results from an unequal sister chromatid exchange. The rate of duplication might have been originally underestimated in the past due to technical reasons, but improved detection techniques now consistently identify duplications in 10–15% of all DMD patients.

Regarding the methods for detecting deletions and duplications, most diagnostic laboratories currently use a system of multiplex PCRs, in which several exons are co-amplified in the same reaction tube, or MLPA (see Chapter 4). The multiplex PCR technique takes advantage of the fact that deletions and duplications almost invariably involve two regions of the gene (the 5' and the 3' deletion 'hot spots'); not all 79 exons need to be analysed for identifying mutations. In practice, by studying only 19 exons in two multiplex PCR assays, ~98% of deletions can be identified. This test has the advantage that it can be run in hours. In addition, it can be relatively easily used under quantitative conditions, therefore allowing the dosage of amplified DNA to be established, an important point in carrier studies and in duplication detection. A major disadvantage of the multiplex PCR assay occurs when the deletions endpoint fall outside the region covered by the 19 exons studied. In these cases, the determination of the effect of the deletion/duplication on the reading frame is impossible. Because of this, MLPA is now one of the most commonly used techniques for the detection of deletions and duplications.

The remaining cases are secondary to point mutations, microdeletions, and intronic rearrangements and splice site mutations. Several methods have been used to look at point mutations and other small gene rearrangements and have



been discussed in Chapter 4. Sanger sequencing of the dystrophin gene and now also the availability of custom exome arrays are methods used for these latter mutations.

Origin of deletions

The precise mechanism responsible for deletions is not known. However, the effect of environmental mutagenic agents might well play a role in this, as has been demonstrated in animal models. The occurrence of deletion hot spots is intriguing, as it suggests that the size of the gene is not the only factor involved. The role of repetitive elements (LINE-1; Alu sequences) has been implicated in specific rearrangements (Suminaga *et al.* 2000). For example, the sequencing of the breakpoint in patients with deletions of exons 2–7 has identified the presence of an Alu sequence in intron 1, 25 kb downstream from the 3' end of exon 1, that was joined directly to a LINE-1 sequence in intron 7, 4.5 kb downstream from the 3' end of exon 7. This novel recombination event therefore joined non-homologous Alu and LINE-1 repeats. Furthermore, the nucleotide sequence of a deletion junction fragment from a DMD patient with a deletion of exon 44 showed that the proximal breakpoint of the deletion in intron 43 fell within the sequence of a transposon-like element, normally present in a complete form in intron 43 of the dystrophin gene (Pizzuti *et al.* 1992).

Considering the frequent occurrence of repetitive elements in the introns of the dystrophin gene, it is likely that they play a role in the origin of deletions. Several palindromic sequences and short tandem repeats, at or near the breakpoints, have been reported (Miyazaki *et al.* 2009). These sequences, with a marked propensity to form secondary DNA structure intermediates, may predispose local DNA to breakage and intragenic recombination in these patients.

Furthermore, the bimodal distribution of deletions within the dystrophin gene (see Fig. 4.13, p. XX) coincided with the bimodal distributions of recombination hot spots. Recent studies indicate that deletion junctions arise from non-homologous ends joining, a major pathway for repairing DNA double-strand breaks in mammals. Mapping of breakpoints in 26 BMD/DMD patients with breakpoints in intron 47 (that is, a major deletion hot spot in the DMD gene) indicated the frequency of breakpoint occurrence around a discrete region being 3-fold higher than expected by chance (Sironi *et al.* 2006).

The dystrophin gene

The dystrophin gene was isolated and characterized by first using PERT and XJ probes to identify relevant mRNA transcripts in human skeletal muscle.

The corresponding cDNA was then cloned and sequenced. The gene proved to be some 3000 kb in length, now known to consist of a total of 86 exons (79 exons, including at least seven promoters) of mean size 0.2 kb and introns of mean size 35 kb. The gene is transcribed into a 14 kb mRNA. Thus, the actual coding sequences represent <1% of the nucleotide composition of the gene locus.

Muscle dystrophin is not the only product of the Duchenne gene locus. There are three full-length isoforms that have the same number of exons as the main muscle (M) isoform, but derived from different promoters (B isoform where B stands for brain, and P isoform for cerebellar Purkinje neurons).

Multiple shorter isoforms also exist, generated from promoters located within the dystrophin gene. These are indicated by their MW and named Dp260, Dp140, Dp116, and Dp71, generated by promoters located in introns 30, 45, 56, and 63, respectively. Several of these isoforms are relevant for the function of dystrophin in the brain and the retina and have been described in detail in Chapter 6.

Dystrophin

Using polyclonal antibodies directed against fusion proteins produced in bacteria from cDNA, the protein product of the Xp21 locus has been identified (Hoffman *et al.* 1987). It has been named dystrophin and, as would be predicted, is a very large protein (see Fig. 9.6).

The main isoform expressed in muscle has an MW of 427 kDa, consisting of 3685 amino acids. It is a rod-shaped protein, composed of four domains:

- ◆ an N-terminal domain with homology to α -actinin and composed of 240 amino acids;
- ◆ a central rod domain, formed by a succession of 25 triple helical repeats, similar to spectrin, and composed of roughly 3000 amino acids;
- ◆ a cysteine-rich domain, composed of 280 amino acids;
- ◆ a C-terminal domain, composed of 420 amino acids.

That dystrophin shares many features with spectrin and α -actinin indicates that it too is a cytoskeletal protein. Early on, it was shown to be localized to

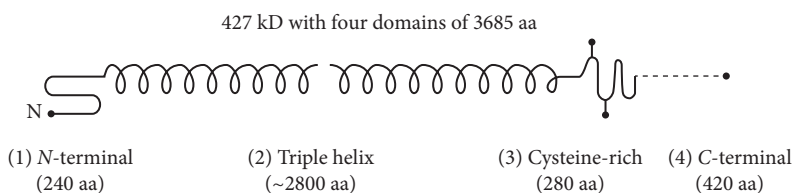


Fig. 9.6 The dystrophin molecule. aa, amino acids.

muscle cell membranes. It is a costameric protein, forming a lattice that encircles the muscle fibre and attaches the sarcomeres to the sarcolemma. Dystrophin is not directly inserted into the membrane but binds β -dystroglycan, a transmembrane glycoprotein that is part of the dystrophin–glycoprotein complex (see Fig. 9.7). The role of this complex in the pathogenesis of muscular dystrophy will be discussed in Chapter 10.

Regarding the shorter dystrophin isoforms, none is expressed in skeletal muscle. They all differ at their *N*-terminus, whilst they share with the full-length isoform the cysteine-rich domain and *C*-terminus. These shorter isoforms therefore all lack the actin-binding domain, whilst they share the β -dystroglycan-, dystrobrevin-, and syntrophin-binding domains, suggesting the potential to interact with similar proteins at their *C*-terminus (see Fig 10.1).

The presence of so many different isoforms and of dystrophin on different specialized membrane surfaces implies multiple functional roles for dystrophin proteins. The nature of these roles is, as yet, not fully understood but is probably relevant in the distribution and extent of involvement of different tissues in DMD and BMD, as a consequence of mutations at the Xp21 locus.

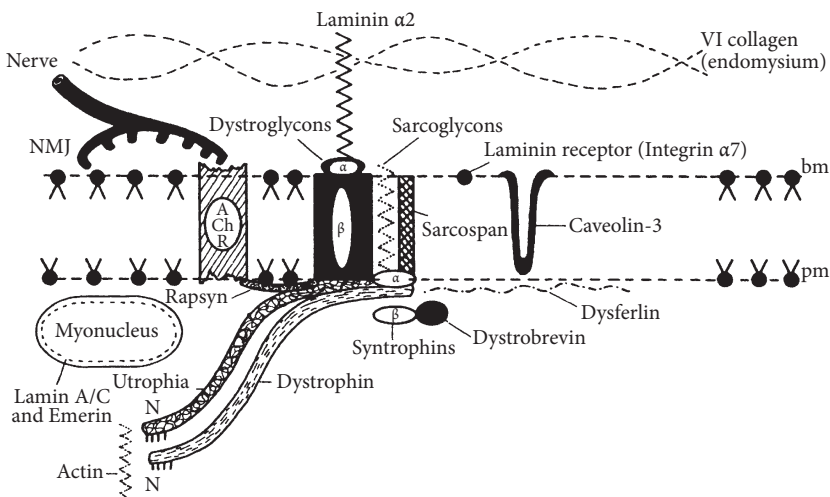


Fig. 9.7 Schematic representation of the various proteins involved directly or indirectly in different forms of muscular dystrophy. The precise relationships of these various proteins is not yet entirely clear. AChR, acetylcholine receptor; bm, basement membrane (basal lamina); NMJ, neuromuscular junction; pm, plasma membrane (plasmalemma).

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Molecular pathology and phenotype

There is no simple relationship between the extent of a mutation and the resultant clinical disease. For example, the deletions of small exons, such as exon 44, or a comparatively minute deletion of 52 bp out of 88 bp in exon 19 result in classical DMD. On the other hand, large deletions, involving nearly 50% of the gene, have been described in patients with BMD. Similarly, a massive duplication of >400 000 bp within the central region of the gene resulted in a dystrophin of ~600 kDa, and yet the patient only had mild BMD. The effects on the phenotype depend therefore not so much on the extent of a deletion/duplication, but on whether or not it disrupts the reading frame.

Frameshift hypothesis

The milder BMD results from mutations that maintain the reading frame (in-frame), resulting in an abnormal, but partially functional, dystrophin, but, in DMD mutations, disrupt the reading frame (frameshift) so that virtually no dystrophin is produced (see Fig. 9.8). This reading frame hypothesis (Monaco *et al.* 1988) holds for ~90% of cases. But exceptions do occur such as mild BMD with a frameshift deletion or DMD with in-frame deletions. It is important to be aware of these cases, as they might lead to diagnostic confusion if the diagnosis is approached only using molecular genetic techniques.

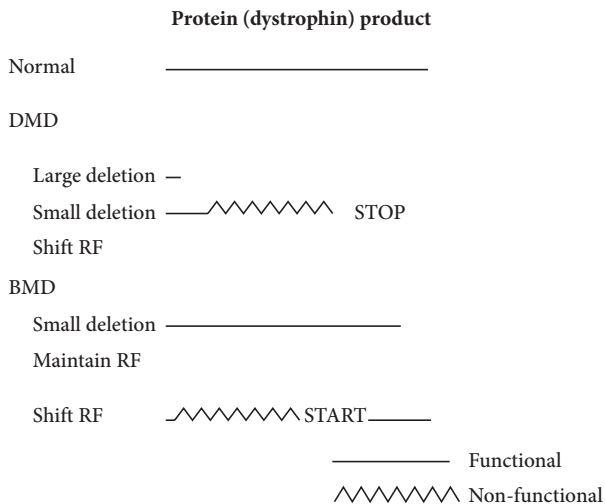


Fig. 9.8 Simplified diagram of the effects of mutations in the dystrophin gene on the gene product that maintain or disrupt (shift) the reading frame (RF).

Regarding cases of BMD with a frameshift deletion, these involve deletions or duplications in the 5' end of the gene (exons 3–7, 5–7, 3–6), but also exons 51, 49–50, 47–52, 44, or 45. In a series of one of the authors (Muntoni), more than half of the patients with out-of-frame deletions of exon 44 or 45 had milder phenotypes than DMD, mostly intermediate cases between DMD and BMD, but also rarely BMD cases. Moreover, rare patients with BMD, despite non-sense mutations (such as single nucleotide changes leading to a non-sense mutation), have also been described. These patients succeed in producing dystrophin by using exon skipping via alternative splicing (see Chapter 13) or in-frame translational re-initiation. Finally, splice site mutations predicted to lead to an out-of-frame deletion, but actually resulting in larger deletions leading to restoration of the open reading frame (ORF), have also been described in BMD.

On the whole, dystrophin amounts on muscle histochemistry or Western blot analysis correlate better with the phenotype than the 'genetic prediction' in patients with exceptions to the reading frame rule. In counselling patients and families, it is therefore important to mention that, whilst a diagnosis exclusively based on genetic studies is certainly less invasive, the accuracy is only 90%. Obviously, this is not relevant when other affected individuals are present in a family or in cases in which the phenotype is obvious (such as, for example, a child with major walking difficulties at diagnosis, aged 5; or vice versa a child with no functional deficit when diagnosed, especially if in the second part of the first decade of life or later). In cases in which the phenotype is not clear yet, because of the young age of the child, and especially in cases with mutations which have been previously associated with variable phenotypes, our recommendation is to consider performing a muscle biopsy to study the expression of dystrophin on muscle histochemistry in order to avoid a misdiagnosis.

There is, however, some controversy as to whether the dystrophin level helps in predicting the severity of BMD. Some authors have commented on the lack of a simple correlation between the dystrophin quantity and phenotype, once all BMD patients are analysed together. On the other hand, when BMD patients with identical deletions in the 3' hot spots are grouped together, the severity of the phenotype broadly correlates with the amount of dystrophin produced. This might also depend on the different dystrophin proteins produced in BMD patients with different deletions. In BMD with deletions of exon 45–51, 48–51, or 45–53 and 52–53, there is a good correlation between the amount of dystrophin produced and the resulting clinical course (Anthony *et al.* 2011). On the other hand, this correlation could not be seen in BMD with deletions located more towards the 5' such as 45–47 or 45–48. The dystrophin level of this second group of patients was much lower, nearly half, compared to BMD with deletions

in exons 45–51, 48–51, 45–53, and 52–53, indirectly suggesting differences in stability of the various internally deleted dystrophins.

In an extensive study of patients with DMD with frameshift mutations, the size of muscle dystrophin, detected at greatly reduced levels, agreed closely with that expected, if the reading frame were restored and the translation proceeded. Even the very low levels of muscle dystrophin in DMD may therefore have some functional significance. There is a correlation between the amount of dystrophin in these cases and the severity of the disease, as assessed by the age at becoming confined to a wheelchair, although it is not clear how much of the phenotypic variability observed in DMD could be attributed in this way. In the personal series of one of the authors (Muntoni), deletions associated with a higher level of residual dystrophin expression (for example, exons 44 and 45 deletions) were associated with a slightly less severe phenotype, compared to deletions in which only a minimal level of residual dystrophin expression can be detected (for example, exon 53 deletions). Using functional scales, such as the North Star scale for functional abilities, the latter group of patients decline at a statistically significant faster rate than those DMD patients with deletions of exon 44 or 45.

Exceptions to the reading frame rule occur not only in BMD, but also in DMD. It is worth mentioning them, as they may cause diagnostic confusion. These patients typically have in-frame deletions that are nevertheless associated with a severe phenotype. In particular, most of these patients have relatively large deletions in the 5' region, extending into the mid-rod domain, for example, deletions removing exons 3–31 and 3–25, 4–41, 4–18, and 3–13. These deletions involve the 5' principal putative actin-binding site of dystrophin, and they are therefore likely to directly affect the interaction of dystrophin and actin or affect it indirectly, following an abnormal folding of dystrophin (Norwood *et al.* 2000). In-frame deletions removing the β -dystroglycan binding domain (exons 63–70) also result in DMD, irrespective of the amount of dystrophin protein present.

Mutation site

Although deletions (and occasional duplications) can occur almost anywhere in the dystrophin gene, most occur in two 'hot spots'. These have been referred to as the central high-frequency deletion region (HFDR) and the proximal HFDR (see Fig. 4.12). The central HFDR is located ~1200 kb from the 5' end of the gene, clustered around exons 45–55. The proximal HFDR is located ~500 kb from the 5' end of the gene, clustered around the first 20 exons. The clusters of these two hot spots represent the basis for using the multiplex PCR technique, which, by screening only 19 of the dystrophin exons, identifies ~98% of all deletions.

Various authors have shown that proximal (5') deletions are more common in familial cases, and distal deletions in isolated cases. A 'proximal' new mutant has a greater risk of recurrence of about 30%, and a 'distal' new mutant has a lesser risk of recurrence of about 4%.

The majority of out-of-frame (frameshift) deletions in these regions, as well as elsewhere, result in severe DMD. Most of these cases have virtually no dystrophin. A few missense mutations, with residual expression of dystrophin, have been described in patients with DMD. The most convincing of these was reported in exon 69, in the β -dystroglycan binding site. This patient followed a severe DMD course, despite the residual production of dystrophin, further reinforcing the importance of the interaction between β -dystroglycan and dystrophin for the function of the complex (Lenk *et al.* 1996).

A most interesting finding is that in-frame deletions of the central region of the gene that remove almost 50% of the dystrophin can result in a mild phenotype. The resultant significantly truncated dystrophin is therefore adequate for almost normal muscle function. Furthermore, some deletions within this region may produce myalgia and muscle cramps, but no weakness, or even no symptoms at all or merely a raised SCK level. We have studied three multigeneration families, in whom an in-frame deletion of the central rod domain of the dystrophin gene, involving either exons 32–44, or 48–51, or 48–53, was only associated with an elevation of SCK.

Rare and unusual dystrophin gene mutations have also been associated with XLDCM. This condition is a disease of heart muscle, in which skeletal muscle is clinically not affected, although slight elevation of SCK has been documented in several affected families. Affected males develop symptoms of cardiac failure in the second or third decade of life. Cardiac transplant may be the only therapy possible in some severely affected males. We now know that this condition, initially mapped to the same locus as DMD and BMD, is a rare allelic variant of these two disorders. Most of the reported mutations occur in the 5' region of the gene (see Table 9.2) and selectively affect the expression of dystrophin in the heart. Detailed characterization of these cases may eventually clarify the differences in dystrophin transcriptional pathways between skeletal and cardiac muscle (for a review, see Ferlini *et al.* 1999).

These data indicate that there is a wide spectrum of abnormalities associated with mutations at the Xp21 locus. In fact, there may be healthy males in the population who have small defects in the central rod region of the dystrophin protein that may only be expressed under stress such as muscle cramps after exercise or excessive muscle fatigue in later life. We are starting to understand the relationship between the effects of different mutations within the Xp21 locus and the cardiac function. For example, a mutation that does not cause

Table 9.2 Mutations of the dystrophin gene in XLDCM

Dystrophin gene mutation	Age of patients at study or death	Prognosis	CK
Muscle exon/intron 1 junction deletion	13	Death	High
Muscle exon 1 3' splice site point mutation	24	Death*	Normal
Muscle exon 1 3' splice site point mutation	11	Death	High
Splicing point mutation in intron 1 of muscle transcript	12	Death	High
Insertion of L1 element in muscle exon 1	17	Death	High
Insertion of L1 element in muscle exon 1	10	Death	High
Exons 45–48 deletion	39	Alive	Normal
Exons 45–55 deletion	36	Alive	High
Exon 48 deletion	17	Alive	High
Exon 48 deletion	24	Alive*	Normal
Exons 48–49 deletion	24	Alive	High
Exons 48–51 deletion	30	Alive	High
Exons 48–53 deletion	50	Alive	Normal
Exons 49–51 deletion	50	Death*	Normal
Exons 2–7 duplication	20	Alive*	High
Exon 9 point mutation	21	Death	High
Intron 11 deletion	16	Death*	High
Exon 29 point mutation	21	Death*	High
Exon 35 missense mutation	12	Death	High

* Heart transplantation.

weakness might conceivably affect the cardiac muscle in some way in later life in an otherwise healthy individual. We demonstrated this in two cases of XLDCM with 'typical BMD deletions', involving exons 48–49 in one case and exons 49–51 in the other. Intriguingly, SCK levels were entirely normal in this latter case.

Summary and conclusions

Several females with DMD and X/autosome translocations have been described, the breakpoint on the X chromosome always being at Xp21. This, and the fact that a unique case of DMD was found with a microscopically visible deletion in this same region of the X chromosome, pointed to the disease locus

being located at this point. Whilst, in the past, a number of different techniques were used to trace the affected X in cases with no clear mutation identified (for example, RFLPs which are polymorphisms due to the presence or absence of a particular restriction site, or microsatellite polymorphic markers and SNPs around Xp21 and linked to the Duchenne locus), nowadays the use of newer techniques allow the identification of mutations in virtually all DMD cases and has improved the accuracy of carrier detection in families.

The analysis of the dystrophin gene, initially by either Southern blot or multiplex PCR, but now almost universally by MLPA, is able to identify submicroscopic gene deletions in up to 70% of cases of DMD and BMD and duplications in 10–15% of cases. The remaining cases carry point mutations that are spread throughout the entire gene, and their detection requires direct sequencing of the entire coding region of the gene, which is technically demanding.

Finally, the Duchenne gene has been cloned and studied, and it has proved to be at least 3000 kb in length and to consist of at least 86 exons when the unique first exons of the internal isoforms are also considered. The gene product has been identified and called dystrophin. This is a very large protein, which is present in only very small amounts in normal muscle. It is virtually absent or non-functional in muscle in patients with DMD, and internally deleted, presumably semi-functional, in BMD. It is associated with muscle cell membranes via glycoprotein, forming a dystrophin–glycoprotein complex. Disruption of this complex, consequent to a deficiency of dystrophin, initiates the train of events that ultimately leads to muscle cell death. Dystrophin exists in a number of different isoforms with different tissue distributions. The effects of mutations at the Xp21 locus on these dystrophin isoforms might well explain the distribution and extent of involvement of different tissues in DMD.

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Chapter 10

Pathogenesis

Introduction to pathogenesis

When this book was first written in the mid 1980s, much space was given to an examination of the evidence for what were referred to as the vascular, neurogenic, and membrane hypotheses of causation. But, with the discovery that the primary cause was a deficiency of dystrophin located at the cell membrane, there seemed no longer any reason to consider much previous research on the subject. However, among these previous research findings, there may be important ideas necessary for a full and complete understanding of the disease process. Thus, although a vascular or neurogenic defect is definitely not the primary cause, nevertheless, the contribution of a not entirely adequate blood supply to muscle might aggravate the disease process. Proper functioning and organization of the neuromuscular junction are also essential for the correct differentiation of the muscle fibres. We do not know at the moment if such influences, in addition to a deficiency of muscle dystrophin during muscle development, might contribute to the full expression of an abnormal phenotype. With the recent advances in our understanding of the cellular partners of dystrophin, a complex pathogenic scenario of its functions has emerged. These functions will be discussed in this chapter and are summarized in Box 10.1.

Evidence in support of a mechanical role for dystrophin

Protein structure

Sequence analysis of the dystrophin gene has, from the outset, attributed to dystrophin a structural role. The *N*-terminal first ~246 amino acids of dystrophin contains the main actin-binding region, which shows significant sequence homology to a number of structural molecules that include α -actinin and spectrin, whilst the cysteine-rich domain contains the binding site for β -dystroglycan. The importance of these two regions for the appropriate function of the protein, sometimes described as a 'shock absorber', can be inferred by the observation that even small in-frame deletions in these two regions, for example, exons 3–13, 3–15, 4–18, or exons 61–69, or 66–68, often result in

Box 10.1 Summary of cellular functions proposed for dystrophin in muscle

Mechanical role

Stabilization of the membrane by linking to F-actin and β -dystroglycan
Force transmission

Cell signalling

Syntrophin and dystrobrevin
Grb2 (growth factor receptor-bound 2), a signal-transducing adaptor protein (via β -dystroglycan)
Calmodulin (regulator of calcium-dependent kinases)
Serine/threonine kinases (via β 2-syntrophin)
nNOS
Stretch activated channels
RYR1 nitrosylation

Others

Calcium homeostasis
Role in smooth muscle
GLUT4 translocation
Regeneration and fibrosis
Immune factors
Neurogenic abnormalities

a DMD phenotype. These observations, together with those made in transgenic mouse models, strongly suggest that interactions between dystrophin and the F-actin associated cytoskeleton towards the *N*-terminus of the molecule and β -dystroglycan towards the *C*-terminus are of considerable functional relevance (see Fig. 10.1).

The *N*-terminal domain is followed by a large region, consisting of 24 spectrin-like triple helical repeats that form the **rod domain**. This region of dystrophin also contains an actin-binding site (between spectrin repeats 11 and 14) that is not present in the utrophin rod domain. The functional relevance of this difference is not yet understood. In-frame deletions in the central rod domain are often associated with the milder BMD, suggesting that, from a functional perspective, this domain is not crucial. In some BMD patients, these deletions can

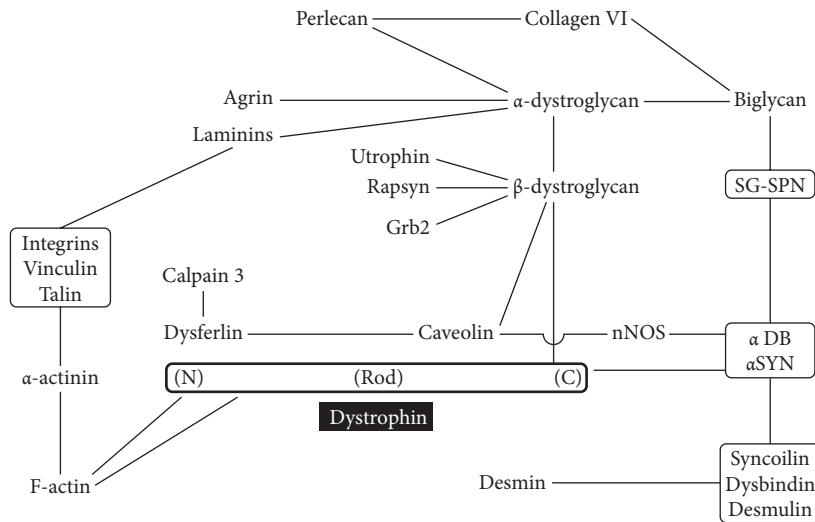


Fig. 10.1 Proposed binding interactions of various muscle proteins.

Adapted from *Neuromuscular Disorders*, Volume 12, Issue 4, Emery, A. E. H., *Muscular dystrophy into the new millennium*, pp. 343–9, Copyright © 2002, with permission from Elsevier.

be extremely large, for example, as the case of a mild BMD with a deletion removing exons 17–48. The functionality of this shortened dystrophin has been further confirmed by transgenic experiments with the engineering of *mdx* mice that express a construct with the same deletion, resulting in marked amelioration of the pathology. These observations are of considerable importance, with respect to attempts to develop a gene therapy for DMD using truncated versions of dystrophin, since it is extremely challenging to pack a viral vector with the full-length 14 kb dystrophin cDNA, in addition to a promoter. The capacity of even shorter carefully engineered isoforms to rescue the pathological phenotype in *mdx* mice has been further assessed by Chamberlain's group, using both transgenic and gene therapy approaches. The results obtained with these experiments are encouraging (Harper *et al.* 2002).

Immediately after the rod domain is the **WW domain**, which separates the rod from the **cysteine-rich** domain. The WW is a motif found in several proteins with regulatory and signalling functions. This region is involved in the interaction of dystrophin with β -dystroglycan. This link is critical for the functional integrity of the dystrophin complex, as mentioned before. β -dystroglycan is a pivotal protein of the DAPC—it binds α -dystroglycan, a highly glycosylated receptor for laminin $\alpha 2$ chain and other extracellular matrix proteins, thereby mediating a link between the cytoskeleton and the extracellular matrix; in

addition, β -dystroglycan binds to sarcoglycan proteins, responsible in their own right, when defective, of muscular dystrophy variants which share similarities with DMD (sarcoglycanopathies, previously described as DMD-like muscular dystrophies).

The cysteine-rich domain contains sequence motifs involved in various interactions. These are two EF-hand motifs (involved in calcium binding), followed by a ZZ domain that binds to calmodulin in a calcium-dependent manner. Adjacent to the cysteine-rich domain is an alternatively spliced region and two coiled-coil motifs that form the C-terminus of dystrophin. The alternatively spliced region binds syntrophin, whilst the coiled-coil motifs bind dystrobrevin. This coiled-coil region is arranged in an α -helical manner, similarly to the rod domain. The cellular functions of dystrobrevin and syntrophin have yet to be established. However, various lines of evidence suggest that these molecules are involved in cell signalling. In particular, syntrophin binds to nNOS, sodium channels, stress-activated protein kinase-3 (SAPK-3), and a microtubule-associated serine/threonine kinase.

Localization of dystrophin in skeletal muscle

Dystrophin is not distributed evenly on the inner side of the sarcolemma but appears to be enriched at the neuromuscular and myotendinous junctions and the costamers.

The **costamers** were first described in 1983 as discrete subsarcolemmal vinculin-rich domains that flank the Z-disc and link the contractile apparatus to the sarcolemma, along the entire length of the fibre. They have since been shown to be composed of a number of cytoskeletal proteins that include dystrophin, talin, spectrin, ankyrin, and desmin. This arrangement facilitates the lateral transmission of force and alignment of the sarcomers, which may be particularly relevant to fibres that do not run from one end of the muscle to the other, but rather terminate in the perimysium.

At the **neuromuscular junction**, the muscle fibre membrane is thrown into a series of folds, with the crests of each fold adjacent to the nerve terminal and containing the acetylcholine receptors. Dystrophin and utrophin occupy distinct domains within this region, with dystrophin and the voltage-gated sodium channels being found at the base of each fold, and utrophin co-localizing with the acetylcholine receptors at the crests. The muscle fibre membrane is also highly folded at the myotendinous junction. A comparison of *mdx* mice with those deficient in integrin $\alpha 7$ - $\beta 1$ suggests that, whilst integrin $\alpha 7$ is the major receptor connecting the muscle cell to the tendon, the dystrophin-glycoprotein complex is necessary for the lateral integrity of the muscle cell in this region.

In the absence of dystrophin at the myotendinous junction, other cytoskeletal proteins, such as talin and vinculin, are upregulated.

Sarcolemmal integrity and contraction-induced muscle injury

It is difficult to be sure when the idea that a significant defect in DMD might reside in the muscle cell membrane was first considered. Certainly, this possibility was put forward in the 1950s when several muscle enzymes were found to be increased in the serum of affected boys. Further evidence came in the mid 1970s when electron microscopy identified sarcolemmal defects in the muscle of children with DMD (the so-called delta lesions). More recently, numerous studies have shown that dystrophin-deficient *mdx* mouse muscle fibres display an increased damage when subjected to eccentric contractions.

Even normal skeletal muscle is susceptible to mechanical damage, particularly during lengthening contractions (eccentric exercise), with immediate defects at the sarcolemma and other proteins of the contractile apparatus, which determine a transient decrease in the isometric force. Following repetitive lengthening contractions, there can also be considerable damage. Nevertheless, in normal muscle, the injury can be fully repaired by active muscle fibre regeneration from satellite cells.

Several independent studies have shown that *mdx* mouse muscle is highly susceptible to lengthening contraction-induced injury. Muscle from *mdx* animals demonstrate an increased tendency to take up Procion® orange dye, which suggests an increase in sarcolemmal permeability, following contraction. When injected into *mdx* animals, the Evans blue dye is also selectively taken up by muscle fibres that appeared necrotic, suggesting a link between increased membrane permeability eventually and cell death. Muscle from *mdx* mice also demonstrated a significant decline in force generation, following eccentric contraction, both *in vitro* but also *in vivo*, following specific physiological testing or even prolonged running downhill.

Such work identifies an increased susceptibility to dystrophic membrane damage, relative to normal muscle. In the absence of dystrophin, the sarcolemma is more susceptible to damage by contractile forces, resulting in increased permeability of ions and small molecules, and continuous cycles of muscle degeneration that eventually cannot be compensated by the regenerative capacity of the muscle stem cells. This results in the replacement of muscle by connective tissue and fat.

Dystrophin-deficient mouse myotubes also appear to be more susceptible to osmotic damage and display a substantial reduction in cortical stiffness, relative to controls, further supporting this view.

Linkage with the extracellular matrix

α -dystroglycan and integrin $\alpha 7$ are the two main receptors for laminin $\alpha 2$ in striated muscle (see Fig. 10.1). Integrin $\alpha 7$ is upregulated in DMD and *mdx* muscle. The transgenic overexpression of integrin $\alpha 7$ in mice deficient in both dystrophin and utrophin results in the amelioration of some aspects of their phenotype and the prolongation of their lifespan. These observations emphasize the importance of maintaining a linkage between the basement membrane and the cytoskeleton of the muscle fibre. A number of transgenic experiments in several different animal models support this concept. For example, the high expression of a utrophin transgene in *mdx* mice almost totally restores normal muscle function. The overexpression of an agrin minigene in the *dy/dy* mouse, which is deficient in laminin $\alpha 2$, leads to an amelioration of the dystrophic phenotype by the stabilization of α -dystroglycan and laminin $\alpha 4$ and $\alpha 5$ chains. Finally, the overexpression, at the sarcolemma, of the enzyme CT GaINAc transferase, normally located in the synapses, induces an increased expression of dystroglycan, utrophin, and other components of the dystrophin-associated protein complex at the muscle fibre. The upregulation at the sarcolemma of these synaptic proteins significantly reduces the muscle pathology of *mdx* mice.

Muscle proteins and acquired infections

Apart from the binding of α -dystroglycan (α DG) to various extracellular proteins, it also acts as a receptor for various pathogenic viral and bacterial microorganisms—a function which depends on effective glycosylation. Reduced glycosylation of α DG by mutations of glycosyltransferases could therefore possibly enhance immunity to certain pathogens. In fact there is now clear evidence that glycosyltransferase mutation LARGE (responsible for MDC1D) is critical for α DG to function as a receptor for lymphocytic choriomeningitis, Lassa fever and other arenaviruses (Kunz *et al.* 2005). Such a mechanism may have maintained particular mutations within certain populations (Emery 2008).

There is also evidence from laboratory studies that particular muscle proteins are involved in the pathogenesis of several acquired infections (Emery 2001).

Evidence in support of a signalling role for dystrophin

Dystrophin is part of a complex of glycoproteins (DAPC), some of which have been shown to have a clear signalling role. Since several of these proteins are significantly affected by dystrophin deficiency and some have also been primarily involved in other muscular dystrophies, it is conceivable that their secondary deficiency in DMD is involved in disease pathogenesis. The proteins more closely associated to the DAPC are shown in Fig.10.1.

For example, β -dystroglycan binds to Grb2 (growth factor receptor-bound 2), a 25 kDa protein that participates in signal transduction pathways involving receptor tyrosine kinases. Many of the signalling pathways associated with the integrin axis also involve Grb2, raising the possibility that some of the signalling pathways activated by the integrins may be shared by dystroglycan.

Caveolin 3, the protein of which a deficiency causes a form of autosomal dominant LGMD (LGMDIC) (see Chapter 2), has been shown to compete with dystrophin for the β -dystroglycan binding site. Mice overexpressing caveolin 3 show an apparent downregulation of dystrophin and β -dystroglycan, together with more severe pathological changes than those normally observed in *mdx* mice. Overall, these observations imply that the interaction between dystrophin and dystroglycan is likely to be highly dynamic.

Syntrophin also possesses the capacity to bind to Grb2, in addition to voltage-gated sodium channels, nNOS, and SAPK-3. The dynamics of the syntrophin–dystrophin interactions appear to be regulated by calmodulin and by the phosphorylation or dephosphorylation of dystrophin. Syntrophin itself is phosphorylated by a calcium–calmodulin-dependent protein kinase, that regulates its binding to dystrophin, and by SAPK-3. Mice deficient in α 1 syntrophin display abnormalities of the neuromuscular junction, with secondary reductions of utrophin, acetylcholine receptor, and acetylcholinesterase, emphasizing the importance of syntrophins in recruiting and stabilizing proteins involved in signalling at the sarcolemma. Dystrophin also has several potential phosphorylation sites in the C-terminal region and is the target of a variety of kinases, including calmodulin-dependent protein kinase II, p44^{erk1} mitogen-activated protein kinase (MAPK), p34^{cdc2} kinase, and casein kinase. The phosphorylation of dystrophin has been found to alter its affinity both for actin and syntrophin *in vitro*. Whilst the functional significance of these phosphorylation events *in vivo* still has to be demonstrated, nonetheless, they do provide possible mechanisms by which the dystrophin–glycoprotein complex can be dynamically regulated.

The **dystrobrevins** are a family of dystrophin-related proteins with homology to the C-terminal region of dystrophin. The presence of several tyrosine kinase consensus sites in the C-terminal region of certain dystrobrevin isoforms suggests that protein–protein interactions may be modulated by tyrosine phosphorylation. Further evidence, in favour of a role for the dystrobrevins in cell signalling, comes from the α -dystrobrevin knockout mouse, which develops a mild dystrophy but fails to show any overt disruption of the DAPC. A possible mutation in the α 1 dystrobrevin gene has been found in a single family with dilated cardiomyopathy. Further mutations will have to be identified before definitively assigning a role for α 1 dystrobrevin in dilated cardiomyopathy.

Secondary deficiency of neuronal nitric oxide synthase: modulation of blood flow and immune response

Dystrobrevin binds to the C-terminus of dystrophin and, together with syntrophin, forms a ternary complex that is responsible for localizing nNOS to the muscle fibre membrane. DMD patients and *mdx* mice fail to localize nNOS to the membrane. Mice in which the genes for either $\alpha 1$ dystrobrevin or $\alpha 1$ syntrophin have been knocked out also fail to properly localize nNOS. However, only the $\alpha 1$ dystrobrevin knockout animals display any signs of a muscular dystrophy. Since no gross histological changes are evident in either $\alpha 1$ syntrophin or nNOS knockout mice, the membrane localization of nNOS was initially not considered to be an important factor in the pathogenesis of DMD. However, sympathetic nervous input to the muscular vasculature causes vasoconstriction that is modulated differently in muscle at rest and during exercise. Various lines of evidence now indicate that the inhibition of sympathetic vasoconstriction in contracting muscle is defective in mice with a deficiency of nNOS at the sarcolemma. An abnormal regulation of blood flow within exercising skeletal muscle has also been documented in DMD patients. The vasoconstrictor response (measured as a decrease in muscle oxygenation) to reflex sympathetic activation is not attenuated during exercise of dystrophic muscles. In contrast, this protective mechanism is intact in healthy children and other disease controls, in whom there is no loss of nNOS from the membrane. These observations are of particular interest when considering that necrosis often takes place in groups of fibres in both *mdx* mice and DMD patients, a feature that has often been interpreted as indicative of ischaemia, and thus a possible vascular problem.

The precise domain of the dystrophin involved in the localization of nNOS was recently identified; whilst this is not relevant in DMD, as all patients lack dystrophin and mislocalize nNOS, it is important for BMD patients, as, depending on the type of deletions, nNOS sarcolemmal localization can be either abolished or not.

The loss of sarcolemmal nNOS has also other effects. In particular, it results in increased cytosolic NO production, which determines a series of pathological intracellular events. One of interest is the hypernitrosylation of the sarcoplasmic reticulum calcium release channel ryanodine receptor type-1 (RYR1), now well demonstrated both in (some) BMD and DMD patients. This, in turn, determines spontaneous sarcoplasmic reticulum calcium leakage, which results in impaired muscle contraction (Bellinger *et al.* 2008; Gentil *et al.* 2012).

Other dystrophin functions

Calcium homeostasis

The possibility that increased intracellular calcium might be a significant factor in the pathogenesis of DMD was put forward more than 20 years ago. The

reason for this increase was unclear, although it was recognized that increased intracellular calcium could account for muscle necrosis through the enhancement of calcium-activated proteases. Various techniques (histochemical, biochemical, and electron microscopic) seemingly supported this by showing that the total calcium content was elevated in muscle in DMD, even from a very early age (see Chapter 4), as well as in BMD. Whilst these features were not specific to dystrophin-deficient muscle, early studies suggested these to be more severe in DMD, compared to other neuromuscular disorders. A significant increase in the number of calcium-positive fibres in male fetuses at risk for DMD has also been reported.

Not all of these earlier studies, however, could distinguish the intracellular component of the total calcium. This could only be accomplished several years later, following significant methodological advances. With the advent of fluorescent indicators, it became possible to measure free intracellular calcium $[Ca^{2+}]_i$, and so to evaluate the calcium dynamics of dystrophin-deficient muscle fibres and myotubes *in vitro*. Unfortunately, rather than clarifying the situation, the results obtained so far have proved to be controversial. At least, part of the reason for this may be secondary to subtle methodological differences among the techniques used or the preparation of the tissues. For example, muscle fibre preparation or culture conditions can vary markedly between laboratories, and this might affect results. Furthermore, all the evidence suggests that dystrophin function relies on the maintenance of a link between the cytoskeleton and the extracellular matrix, and isolated fibres and cultured muscle may not prove to be entirely reliable models in which to evaluate the role of the dystrophin axis on $[Ca^{2+}]_i$ handling. It should also be noted that, as a consequence of the behaviour of the fluorescent indicators themselves, it has not been possible to measure $[Ca^{2+}]_i$ immediately under the membrane, which would seem to be most relevant in the case of dystrophin deficiency. Nonetheless, attempts to overcome this have been made recently by using plasma membrane calcium-activated K^+ channels as subsarcolemmal calcium probes. This work suggests that, in the absence of dystrophin, there is, in fact, a higher subsarcolemmal concentration of $[Ca^{2+}]_i$. The development of membrane-localizing fluorescent probes for $[Ca^{2+}]_i$ should enable this aspect to be further investigated in the future.

Another line of enquiry has included the analysis of sequestered calcium using organelle-specific probes. Skeletal muscle is necessarily efficient at controlling large transient fluctuations in $[Ca^{2+}]_i$, and any abnormal influx in calcium may be rapidly sequestered into intracellular compartments such as the mitochondria. This is supported by the observation that the mitochondria of *mdx* myotubes show greater increases in calcium, following potassium

chloride-induced depolarization, than controls. Interestingly, these differences were found to precede alterations in either the cytosol or the cytoplasmic region beneath the membrane, which themselves only became significant at a later stage of myotube differentiation. Taking into account the key role played by mitochondrial calcium handling in cell death, these data suggest that mitochondria are potential targets of impaired calcium homeostasis in muscular dystrophy. Indeed, this may explain why alterations in cytosolic calcium are not always evident, despite the wealth of data from electrophysiological measurements that indicate an increase in calcium leak activity, particularly within the vicinity of artificially induced membrane tears. Since the activity of these channels is apparently decreased in the presence of leupeptin, a protease inhibitor, these observations lend weight to the hypothesis that an initial influx of calcium, perhaps due to transient membrane damage, leads to an increase in calcium-activated proteases. These proteases then induce further calcium influx by acting upon calcium leak channels, the result of which is a mitochondrial overload, a reduction in oxidative phosphorylation, and cell death (see Fig. 10.2).

Whilst elevation in intracellular calcium through membrane tears, as a direct result of dystrophin loss, is one of the events, other mechanisms might also play a role. A role for stretch-activated channels has been hypothesized; the fact that drugs that block these channels (for example, streptomycin) result in a reduction in intracellular calcium and an improvement in force production indicates this axis might also be active. The transient receptor potential (TRP) channels might also be implicated in altered calcium homeostasis in dystrophic muscle. Some of these channels are increased in *mdx* muscle, and their inhibition, using genetic approaches, results in the restoration of normal intracellular calcium levels and improved dystrophic pathology in *mdx* mice.

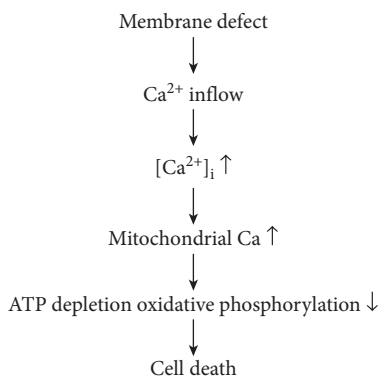


Fig. 10.2 Calcium influx and cell death.

In summary, therefore, the dystrophin-deficient muscle has, on average, an increased calcium content that can be demonstrated with methodologies that are able to measure macromolecular concentrations of calcium.

The calcium content in the cytosol of dystrophin-deficient muscle (non-necrotic fibres) is not grossly abnormal. Calcium might, however, be accumulated in different subcellular compartments such as just beneath the sarcolemma and in the mitochondria.

Whatever the mechanism of the accumulation of calcium (either as a result of specific calcium homeostasis or as a consequence of generalized and gross sarcolemmal damage following contraction), there then follows the enhancement of calcium-activated proteases, as well as mitochondrial overload, resulting in a reduction in oxidative phosphorylation, and eventually cell death (see Fig. 10.2).

Role in smooth muscle

Dystrophin is specifically localized in the caveolae-rich domains of the smooth muscle sarcolemma, together with caveolin. This raises the possibility that the absence of dystrophin in DMD might result in some form of vascular dysfunction. Evidence of abnormalities in the peripheral circulation initially came from early work performed in the 1960s and 1970s by Démos and colleagues in Paris. Since then, it has been found that functional abnormalities of the system are implied by the increased blood loss in children with DMD undergoing spinal surgery. However, detailed studies on the vascular reactivity of dystrophin-deficient *mdx* mice suggest no defect, with the exception of flow-induced dilatation (shear stress), which is decreased by 50–60%. It has been hypothesized that dystrophin could play a specific role in shear stress mechanotransduction in arterial endothelial cells.

Further abnormalities in muscle blood flow have been associated with a deficiency in nNOS.

GLUT4 translocation

Dystrophin contains actin-binding sites at its *N*-terminus, and the distribution of F-actin is significantly abnormal as a result of dystrophin deficiency in the mouse, although such studies have not yet been performed in human muscle. This may be of considerable functional relevance, as one interesting role of the actin cytoskeleton is the recycling of the glucose transporter GLUT4 between the cytoplasm and the sarcolemma. GLUT4 co-localizes with dystrophin, and various studies have demonstrated a deficiency of GLUT4 at the sarcolemma of skeletal and cardiac muscle, in the absence of dystrophin. This observation is paralleled by the biochemical finding of a reduction in glucose transport in the

hearts of *mdx* mice. Furthermore, magnetic spectroscopic data have demonstrated an *in vivo* glycolytic defect in both *mdx* dystrophic mice and patients with dystrophin deficiency. The role of abnormal glucose metabolism in the muscle degeneration seen in DMD is likely to be limited, but perhaps still significant. These observations highlight an important point, namely, that proteins with an obvious structural role may also indirectly contribute to metabolic function.

Regeneration and fibrosis

Myogenic proliferation and differentiation is under the control of growth factors such as TGF- β , insulin-like growth factors (IGFs), and basic fibroblast growth factor (bFGF). In DMD, the chronic accumulation of sclerotic scar tissue in the interstitial space of skeletal muscle is attributed to secondary pathological processes and may itself assume a pathogenic role and contribute to disease progression by interfering with effective muscle regeneration and re-innervation. bFGF enhances the proliferation of skeletal muscle cells *in vitro*, and elevated levels have been reported in the serum of some patients with DMD. bFGF has also been shown to be elevated in necrotic and regenerating muscle fibres of the *mdx* mouse and dystrophin-deficient cats (hypertrophic feline muscular dystrophy, HFMD) where it has been linked to efficient regeneration. This increase is less striking in DMD patients and dystrophic dogs, although whether it accounts for the markedly different phenotypes seen between the two species remains to be demonstrated.

Insulin-like growth factor-1 (IGF-1) has also been shown to stimulate satellite cell proliferation, although its main function is the induction of muscle differentiation. The *mdx* mice carrying a transgene for IGF-1 display a reduction in fibrosis (diaphragm) and necrosis, and an increase in muscle mass and force generation, relative to age-matched *mdx* mice. The overexpression of an IGF-1 transgene has also been reported to ameliorate the early histopathological changes in the *mdx* mouse. These observations underscore the role of growth factors in mediating efficient muscle fibre regeneration. The fact that fibrosis only occurs in DMD and the dystrophic dog, but not in the dystrophic cat or *mdx* mouse, raises some important questions regarding the pathogenetic mechanisms in these different species (see Chapter 7).

Immune factors

The degeneration of muscle fibres in DMD is associated with an invasion of inflammatory cells, such as macrophages and T-lymphocytes, raising the possibility that fibre necrosis may be accentuated by T-cell-mediated injury, as occurs in polymyositis. It has been further suggested that an altered expression of

extracellular matrix ligands and receptors may promote cytotoxic lymphocytes to recognize self as foreign (non-self) and attack the muscle membrane, which then results in the ingress of complement and calcium, with subsequent fibre necrosis. A significant increase in the number of T-suppressor/cytotoxic cells in the peripheral blood in DMD supports this hypothesis. Furthermore, it has been found that HLA class I antigens (major histocompatibility complex (MHC) I) are expressed in skeletal muscle in various muscular dystrophies, but not in normal muscle. This would render the dystrophic muscle more susceptible to T-cell-mediated attack and membrane damage, in addition to any mechanical damage consequent on a defective cytoskeleton. The overexpression of MHC class I antigens is clearly not limited to X-linked dystrophies but represents an epiphenomenon. Nevertheless, it seems reasonable that the expression of a surface antigen that then renders the cell susceptible to T-cell attack has a number of important implications.

Mast cells play a prominent role in inflammation and have been shown to be concentrated in areas of muscle fibre regeneration and necrosis. It has been suggested that the proteolytic activity of mast cells may be responsible for the simultaneous degeneration of large groups of fibres seen in the *mdx* mouse (grouped fibre necrosis). Dystrophin-deficient muscle is clearly more susceptible to muscle fibre necrosis when exposed to intramuscular injections of purified mast cell granules, and an exaggerated pathology is found in *mdx* mice with a heightened mast cell activity. Moreover, a strong correlation between mast cell content and localization and the clinico-histopathological progression in humans, dogs, and mice has been previously demonstrated. Additional data, in support of the role of immune cells in promoting the pathology observed in dystrophin-deficient muscle, come from the observation of increased numbers of activated dendritic cells in dystrophic muscle, relative to controls, that mediate immune responses and probably induce micro-environmental changes.

Another relevant immune factor is NO; this molecule also has an anti-inflammatory and cytoprotective function, and thus its loss from dystrophic muscle could exacerbate muscle inflammation and fibre damage by inflammatory cells. Recent work supports this by showing that the transgenic expression of NOS is able to ameliorate muscular dystrophy in the *mdx* mice. Expression of the NOS transgene in *mdx* muscle also prevented the majority of muscle membrane injury that is detectable *in vivo* and resulted in large decreases in SCK concentrations. The significance of this is shown by the additional finding that *mdx* muscle macrophages are cytolytic, only at the elevated concentrations they are present in dystrophic, nNOS-deficient muscle.

Antibody-mediated depletion of macrophages from *mdx* mice also causes a significant reduction in muscle membrane injury. These findings suggest that

macrophages promote injury of dystrophin-deficient muscle, and the loss of normal levels of NO production by dystrophic muscle exacerbates inflammation and membrane injury.

Neurogenic abnormalities

In addition to muscle, dystrophin is also expressed in several types of neurons, and there is now no doubt that its deficiency in these cells affects the central nervous system and is responsible for some degree of mental retardation (see Chapter 6). The question with which we are concerned here, however, is the possibility that defects at the neuromuscular junction may adversely affect muscle innervation. Many characteristics of fast and slow muscles, including their enzyme profiles and physiological properties, are dependent on appropriate innervation. Furthermore, animal models clearly show that dystroglycan, dystrophin, utrophin, and dystrobrevin are all required for the normal post-natal maturation of the neuromuscular junction. Abnormalities at the neuromuscular junction, in the form of focal atrophy of the post-synaptic regions, have previously been documented in DMD. However, despite these areas of focal degeneration, the amplitude and frequency of miniature end-plate potentials, following nerve stimulation, appear normal. In summary, therefore, whilst subtle defects of the neuromuscular junction might well exist, their role in disease pathogenesis remains to be clarified.

Gene profiling

Using cDNA derived from DMD muscle and hybridizing this on a microarray of DNA sequences of known (and some unknown) genes, it has been found that the transcription of several hundreds of genes appears to be altered in DMD (Noguchi *et al.* 2003; Porter *et al.* 2002). A large number of the observed changes might well have been expected from what is already known of the biochemical changes in dystrophic muscle (see Chapter 7). The study of muscle and serum profiling at different ages suggests stage-specific muscle remodelling—in DMD, inflammatory pathways predominate in the presymptomatic stages, and acute activation of TGF- β and failure of metabolic pathways later in the disease. Nevertheless, this approach does offer an opportunity to possibly identify unknown genes, the transcriptional activity of which is changed in DMD, and some of these might prove to be significant in the pathogenesis of DMD. Indeed, recent studies suggest that polymorphisms in both osteopontin (its gene product has a pro-inflammatory effects in muscle) and LTBP4 (a member of the fibrillin superfamily involved in the binding and sequestration of TGF- β) are modifiers in the DMD course.

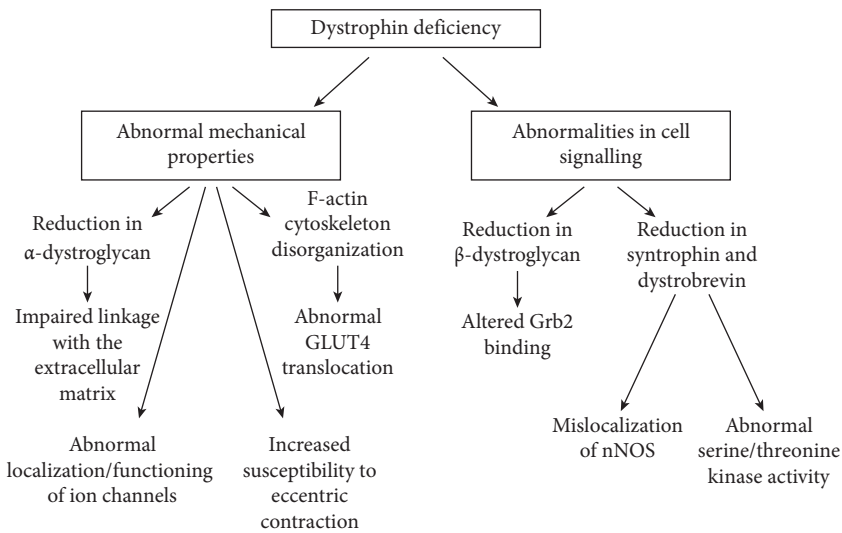


Fig. 10.3 A tentative scheme of the possible pathogenetic pathways in DMD.

Summary and conclusions

The primary defect in DMD is a deficiency of dystrophin, the consequences of which are most profound in striated muscle. Dystrophin is a key member of the dystrophin–glycoprotein complex that, when disrupted, leads to an increase in muscle fibre membrane fragility. This is thought to result in an abnormal influx of calcium, leading to subsequent fibre degeneration. However, additional mechanisms are likely to play a significant role in the pathogenesis of DMD, including defective glucose utilization, blunted vascular response following exercise, increased susceptibility to cytokines, and aberrant cell signalling, the details of which remain to be clarified. Based on current information, a tentative scheme of the possible pathogenetic pathways in DMD has been drawn up (see Fig. 10.3). However, such a scheme, though it may concentrate the mind, gives no indication as to the primary cause of the pattern of progressive muscle weakness and why there is marked differential muscle involvement. The major challenge for the future will be to untangle some of the pathways that give rise to the characteristic pattern of pathogenesis and so open up the prospect of some form of therapy into this seriously debilitating disease.

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Chapter 11

Prevention

Introduction to prevention

Since DMD is a serious disorder for which, at present, there is no effective treatment, a great deal of emphasis has been given to prevention. This involves the ascertainment of women likely to have an affected son and the provision of genetic counselling and prenatal diagnosis for such women.

Ascertainment of families at risk

The ascertainment of women at risk of having an affected child is the first prerequisite of prevention. Logically, this would seem best achieved by screening all females to determine which ones are likely to be carriers. This is impractical, however, because there is, as yet, no simple test that could be used to detect all carriers, and, in any event, such screening would be prohibitively expensive. Having said that, the availability of affordable genetic testing is moving very rapidly forward, and it would not be inconceivable in the future to have a population screening, based on a rapid and inexpensive DMD gene test (preconception diagnosis). Nevertheless, as already observed, in roughly one-third of cases, the mother is not a carrier, the affected son being the result of a new mutation or of a gonadal mosaicism that would not be picked up in such a screening (see Chapter 8).

Another approach might be to screen all pregnancies for affected males. However, quite apart from technical and economic considerations, this would raise a number of serious ethical problems, which were discussed in Chapter 8. At present, the only practical solution is to ensure that all affected boys in the community are ascertained as early as possible and that their mothers, and subsequently other female relatives, are then tested to determine their carrier status and the likelihood of the disorder recurring.

Population screening

Because SCK levels in affected boys are grossly elevated from birth, there is the potential for detection in the neonatal period (see Chapter 8). This can be achieved by determining the SCK level in dried blood spots obtained

from a heel-prick. The blood spots are placed on a filter paper card, and the air-dried specimen can then be assayed immediately or stored. The enzyme activity remains stable for several weeks at room temperature, provided direct heat and sunlight are avoided. Specimens can therefore be conveniently sent by post. With this technique, it is possible to screen newborns for Duchenne. Improvement on this technique are under development, such as the blood spot CK-MM immunoassay which measures the muscle isoform of the enzyme and is expected to be more sensitive than the enzymatic activity. Nevertheless, the ideal test should have a high sensitivity and specificity, an unequivocal predictive value, and a low false positive rate. Unfortunately, the CK test assay does not meet all of these criteria. First, the CK test is a secondary marker for the dystrophic process, that is, an indirect indicator for DMD. Second, a number of false negatives for the CK test assay have been reported in the recent publication from the Welsh programme, that was recently discontinued, following the withdrawal of the external quality assurance scheme by the Centers for Disease Control and Prevention in the US (Moat *et al.* 2013).

Third, the CK test can also produce false positive results, either from birth trauma or due to the presence of other muscular dystrophies (e.g. LGMD), since the test is non-specific for DMD. Although this latter point may be considered a 'bonus' in being able to identify more than one disease from the same test, this raises ethical issues, and regulators insist that the test assay should be specific for the tested condition.

The false positive rate with the various methods of SCK determination ranges between 0.02 and 0.40 (see Table 8.2). When a serum sample yields a grossly elevated SCK level, the diagnosis has to be confirmed by appropriate investigations. There will always be some false positives with this test, because it has to be sufficiently sensitive to detect all cases. It seems likely therefore that the false negative rate among those tested will be low, and only limited to laboratory or administrative errors or failure to test an infant who subsequently develops the disease. Different thresholds for positivity, in order to reduce the frequency of false positive tests, have been suggested. For example, in the study in Ohio, the threshold for considering the test abnormal was raised from 600 U/L (used in a previous pilot phase) to 750 U/L when the study was extended to the entire state, reducing the false positive test rate from 1.6% to 0.52% (Mendell *et al.* 2012). A two-tiered CK/DNA test where, in cases of very high CK, the *DMD* gene is sequenced on the same heel-prick blood spot, confirming the significance of a possible elevation in the first CK (enzymatic) test without the need to proceed with another blood sampling weeks later, is being used in the pilot study from Mendell *et al.* (2012).

However, the important question remains as to whether such screening is really justified. It can be argued that, if an affected boy were detected sufficiently early and his mother proved to be a carrier and was counselled, second cases in the family might be prevented. It has been estimated that up to 15–20% of cases might be prevented in this way. In a series of our patients for whom precise information was available, there were 67 families in which an affected boy was born, but, at the time, no one else was affected in the family and the mother subsequently became pregnant. The average time between the birth of this son and the birth of the next child was 2.71 years (SD, 1.45; range, 1.0–7.0 years). Thus, the next pregnancy was conceived, on average, <2 years after the birth of a son **who subsequently proved to be affected**. Since at least 75% of affected boys present suspicious signs after this age, and the mean age at diagnosis is around 5 years, with a range of 2–8 years, most parents would have been completely unaware of the risks in the next pregnancy. In fact, ten sons in the next, or a following, pregnancy subsequently proved to be affected.

Neonatal screening has also been justified on financial grounds, it being argued that the tests are relatively cheap to carry out and prevention, compared with management, would be cost-effective. However, precise health economic figures of the cost of long-term care on DMD patients do not exist within Europe. Several European-funded projects, such as BURQOL-RD (available at: <<http://www.burqol-rd.com>>; a 3-year project to generate a model to quantify socio-economic costs and health-related quality of life for up to ten rare diseases, including DMD, within Europe), are currently compiling such data. Once available, the cost of a newborn screening programme and its associated long-term care can be compared with other diseases diagnosed following similar schemes and contrasted with their expenses when the diseases are diagnosed in infancy.

Finally, most parents of affected boys questioned in a survey appeared to favour such screening for a number of reasons.

- ◆ It would prevent the anxiety that results from the long delays and unfounded reassurances, often experienced between the first symptoms and the establishment of the diagnosis.
- ◆ Parents have a 'right' to know as soon as possible.
- ◆ It would help prevent further affected children.
- ◆ It has practical advantages in affording an early opportunity to obtain appropriate housing, for example.
- ◆ There are emotional advantages.

However, those questioned in this study were all parents who had already had an affected son. The concern is of presenting a couple with the devastating news that

their newborn son has a serious genetic disorder, when they are completely unprepared for this. This is particularly important, as there are no immediate interventions to apply in the neonatal period that make an interventional compelling case at the moment. Some have therefore advocated a compromise that screening might be restricted to those boys who, at the age of 1 year, receive the vaccinations, or to those who are not walking by the age of 18 months, or when there is a delay in motor and mental development for no obvious reason. The age of 18 months was selected, because, by this time, almost all normal boys, but only about 50% of affected boys, have learned to walk. It has been reasoned that this more restricted screening would have the advantages of involving fewer tests (and therefore lower costs) and that the results would be easier to interpret and less likely to cause anxiety, because the parents' concern is already aroused or subtle signs of the condition are already present. Of course, since the screening would be carried out later, fewer secondary cases (<10%) could be prevented. It would therefore be less effective. It would also be necessary to establish procedures for informing all family doctors of the requirement for testing and the referral of blood samples to an appropriate centre. This could present organizational difficulties.

However, the subject is viewed; the newborn screening for DMD continues to be a controversial issue, and the proceedings of a recent European Neuromuscular Centre (ENMC) workshop, dedicated to the pros and cons of this approach, have recently been published (Ellis *et al.* 2013). There is little doubt, however, that, when an effective treatment eventually becomes available, interest will be rekindled. Importantly, a number of novel therapeutic interventions are currently experimented in phase III studies in the older ambulant DMD population. For it seems probable that the sooner any treatment is begun, the more likely it is to be effective and arrest the course of the disease. A number of issues will then have to be faced, including the very careful and sensitive counselling of parents of proven positive cases.

At present, most paediatricians and geneticists confine their activities to the family of an affected boy. All of his female relatives can be screened in order to assess their carrier status, and records of those found to be at risk can be maintained on a confidential register system for subsequent follow-up.

Carrier detection

The whole problem of genetic counselling in DMD revolves around the detection of female carriers. If all mothers of affected boys were carriers, the situation would be much simpler. This is, however, not the case in approximately one-third of cases, and, in any event, the carrier status of sisters and daughters of carrier mothers often has to be determined.

Definition of carrier status

First, it is necessary to consider the definition of a carrier. In the past, confusion on this point has often led to difficulties in interpreting the results of any proposed tests for detecting carriers. There are three accepted categories of carriers, based on genetic considerations.

- 1 Definite (or obligate) carriers who are mothers of an affected son but who also have an affected brother, affected nephew by their sister, or an affected maternal uncle or other maternal male relative. Included in this category are also mothers of affected sons by different non-consanguineous fathers.
- 2 Probable carriers who are mothers with two or more affected sons, but with no other affected relatives. This could be the result of germline mosaicism (see later) or autosomal recessive LGMD of childhood which clinically resembles DMD.
- 3 Possible carriers who are mothers of an isolated case, as well as their sisters and other female relatives. This category also includes female relatives of definite and probable carriers. The probability of all such women being carriers has to be determined. The term 'suspected carrier' is frequently used for any woman who is at risk of being a carrier.

Biological considerations

The evaluation of carrier detection tests has been bedeviled by several factors. Some of these are inherent in that, DMD being an X-linked recessive disorder, there will inevitably be variability in expression in carrier females because of random inactivation of the X chromosome. This means that a proportion of carriers is unlikely to be detectable by any biochemical or histochemical methods. This is why the use of serum CK, but also of muscle immunohistochemistry, has significant limitations if used in isolation.

Methodological considerations

With improved techniques to assess DNA mutations, essentially all carriers now can be screened with a precise and unequivocal genetic test. Having said that, some of the genetic techniques (such as, for example, cDNA analysis or full gene sequencing) might not be available in all countries. Furthermore, there are rare mutations that can be extraordinarily difficult to be detected. This is why we feel it is still helpful to evaluate the different approaches used to identify a carrier mother.

It should also be remembered that a proportion of carriers may exhibit some degree of muscle weakness—the so-called 'manifesting carriers' (see Chapter 5).

Carrier detection tests

There are essentially three approaches used to detect carriers: SCK, muscle pathology, including immunocytochemistry, and DNA analysis.

Serum creatine kinase

For many years, the most widely used single test for detecting carriers was the SCK level. Following the introduction in clinical practice of the test by Schapira and colleagues in 1960, the detection rate obtained by a number of investigators in the following years was around 60–70% and has not changed significantly since. Its great advantage is its simplicity. Furthermore, the results can be combined with data from linkage analysis to provide valuable additional information for carrier detection. However, in applying the test, possible causes of variation, both in female controls and carriers, must be considered. This variability is partly biological in origin. A slight rise in activity over the course of the day and slightly higher levels in summer, compared with winter, have been reported. However, both diurnal and seasonal variations are small and, from a practical point of view, are relatively unimportant.

The stage of the menstrual cycle and the use of oral contraception have little effect on the SCK level, but vigorous exercise may cause significant increases, though normal daily activity is without any material effect. Age also has to be considered. Several studies have indicated that levels are significantly higher in teenage (especially premenarchal) girls, compared with adult women. Pregnancy is also an important factor, levels being significantly lower in the early stages and significantly higher immediately post-partum where the latter is presumably due to the release of enzymes from the involuting myometrium.

Racial factors may also be involved, since the mean level in black females has been found to be significantly greater than in Caucasian females in the US.

All these various factors also have an effect on SCK levels in carriers. Standardized exercise (on a walking machine or a bicycle ergometer) has been claimed by some to accentuate SCK levels in carriers more than controls, provided that it is strenuous and the effects are followed for several hours afterwards. It has therefore been recommended as a provocative test in suspected carriers with a borderline SCK level.

Some studies have suggested that carrier detection might be better in childhood, but, among daughters of definite carriers who are aged 15 and over and who have not yet had any children, the proportion with SCK levels exceeding the normal 95th percentile (86 IU) for adult women was not significantly different from the expected proportion (31.20%), based on the findings in definite carriers (see Table 11.1).

Table 11.1 The proportion of daughters of definite carriers who have SCK levels that exceed the normal 95th percentile (86 IU) for adult women*

	Number	Age (years)			Proportion	
		Range	Mean	SD	Number	%
Controls	200	18–52	27.06	9.10	11	5.50
Carriers	125	17–70	41.69	11.67	78	62.40
Controls	65	15–20	18.72	0.67	3	4.62
Daughters of carriers	49	15–20	17.33	1.84	16	32.65

* Unpublished data.

SCK levels in carriers may also be affected by genetic factors. This problem has been examined by considering correlations between SCK levels in various female relatives within families of definite carriers (see Table 11.2). All the correlations were positive, but none was significantly different from zero. If there are any familial similarities in SCK levels, these would therefore seem to be relatively unimportant.

In view of the various technical and biological variations that may influence SCK levels, it is therefore not surprising that there is a considerable spread of values in controls, the distribution being positively skewed. In carriers, the spread is even greater and the distribution even more skewed, but there is no suggestion of any bimodality (see Fig. 11.1). Since the distributions in the two groups are so different, results have been expressed as the ratio (h) of normal homozygosity (Y_1) to heterozygosity (Y_2), as shown in Table 11.3. The normal

Table 11.2 Correlations between SCK levels in females within families of definite carriers. The correlations between sisters (daughters of definite carriers) refer to: (1) all sisters; (2) sisters where at least two sisters in a family had SCK levels in excess of 170 IU and are therefore likely to be carriers; or (3) sisters where at least two sisters in a family had SCK levels <86 IU and are therefore unlikely to be carriers*

	Carrier mothers and daughters	Daughters of carriers		
		Sisters (1)	Sisters (2)	Sisters (3)
Number	101	111	20	41
Correlation	0.016	0.138	0.182	0.112
Student's 't'	0.158	1.455	0.785	0.704

* Unpublished data.

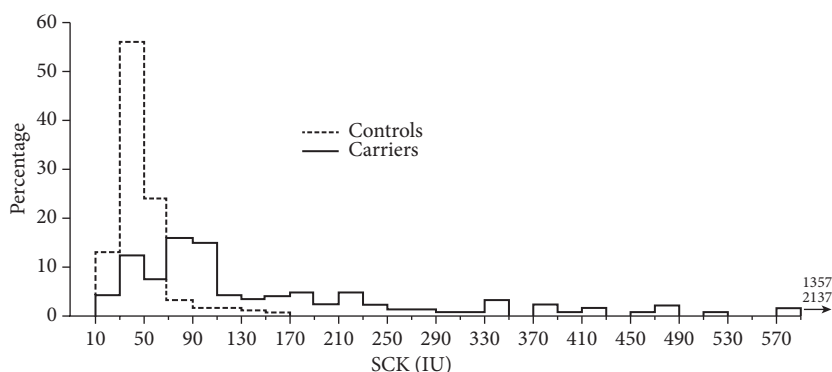


Fig. 11.1 Distribution of SCK levels, determined under standardized conditions, in 200 normal adult control females and 125 definite carriers.

Table 11.3 Distribution of SCK levels in controls and carriers*

SCK (IU)	Controls		Carriers <i>h</i>		(Y ₁ /Y ₂)
	Number	% (Y ₁)	Number	% (Y ₂)	
11–30	26	13.0	5	4.0	3.25
31–50	112	56.0	15	12.0	4.67
51–70	47	23.5	9	7.2	3.26
71–90	6	3.0	20	16.0	0.19
91–110	3	1.5	18	14.4	0.10
111–170	6	3.0	14	11.2	–
>170	0	0.0	44	35.2	–
Total	200	100.0	125	100.0	–

* Unpublished data.

95th percentile (based on the cumulative distribution curve) is 86 IU. Seventy-eight (62%) of definite carriers had levels that exceeded this, and 44 (35%) had levels outside the upper limit of the normal range of 170 IU.

Because of the variability in SCK levels in carriers, our practice and that of others have been, where possible, to take the mean of samples obtained on three separate occasions, in the belief that this might provide a better guide to the carrier status. However, compared with the values obtained with single determinations in controls, no matter how the upper limit of normal is defined, repeat testing seems to have little overall effect on the discriminatory value of the test (see Table 11.4).

Table 11.4 Proportion (%) of carriers ($N = 94$) with SCK levels that exceed the normal upper limit, depending on whether the first, mean, or highest of three determinations is used

	95th percentile (86 IU)	Median \times 2.5 (110 IU)	Median \times 3 (132 IU)
First	58	49	40
Mean	64	46	40
Highest	65	52	41
Controls	5	3	1

Muscle pathology

Different types of abnormalities can be found in the muscle biopsy of carriers, which range from gross morphological changes to subtle abnormalities of dystrophin expression. The muscle biopsy of definite carriers can look entirely normal.

Regarding the morphological changes, these include increased variation in fibre size, eosinophilic 'hypercontracted fibres', increase in internal nuclei, and even fibre necrosis and phagocytosis, although the latter are rare in the absence of florid muscle weakness. In only about 10% of symptomless carriers is there usually any **obvious** abnormality on routine histology. However, careful quantitation of muscle fibre size, internal nuclei, and histochemical fibre type proportions has been claimed to demonstrate abnormalities in around 70% of carriers. These abnormalities can occasionally be detected, even when the SCK level is within the normal range.

Various authors have suggested that, in carriers, there might be a gradual reduction in the number of affected muscle fibres because of their replacement (from proliferating satellite nuclei) by more normal fibres. This may represent the pathological correlate of the described small decline in SCK levels with increasing age in carriers.

Whilst repeated studies of muscle biopsies on DMD carriers have not been performed, several lines of evidence suggest that 'genetic normalization' (that is, the phenomenon by which the repeated cycles of degeneration and regeneration lead to an increase in the number of dystrophin-competent nuclei because of the fusion of dystrophin-positive satellite cells) may occur in carriers (see Fig. 11.2).

The first evidence derives from studies performed in the female carriers of the *mdx* mouse and the dystrophic dog. These studies clearly show that the number of dystrophin-negative fibres declines with age. The pattern of dystrophin expression may also vary, depending on the muscle studied.

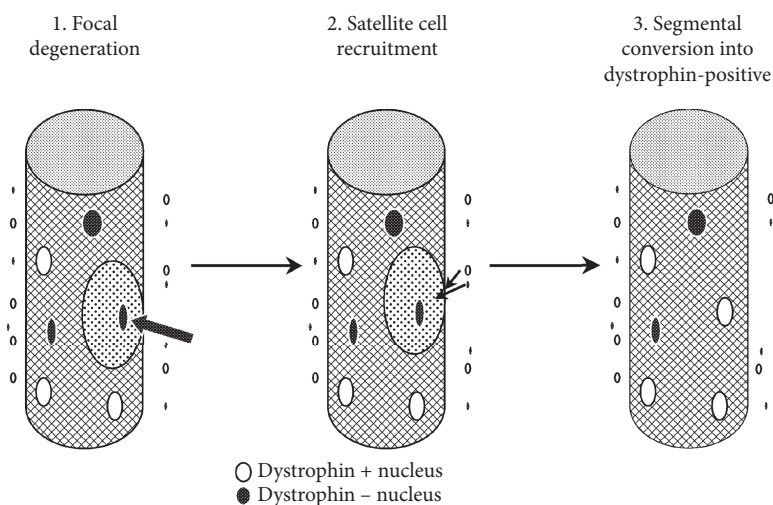


Fig. 11.2 The model of biochemical normalization.

We had the opportunity to study the rectus abdominis of a definite carrier in her sixth decade of life. The rectus abdominis, a non-antigravity muscle that is presumably not significantly affected by dystrophin deficiency (as indicated by the good preservation of its function until late in the course of the disease in DMD), displayed clear clusters of dystrophin-negative and dystrophin-positive fibres of roughly equal proportions. In contrast, the quadriceps of the same carrier expressed dystrophin in almost every fibre. We speculate that the stress of the contraction of the antigravity muscle, with its associated degeneration and regeneration, provided the genetic correction in the quadriceps, whilst the rectus abdominis, in which the degeneration and regeneration were absent, still reflected the original random pattern of X-inactivation.

How do we explain, however, the observation that muscle weakness in manifesting carriers tends to get worse and not improve over the years? A careful study of DMD carriers by Pegoraro *et al.* (1995) addresses this complex scenario. These authors studied not only the expression for dystrophin in muscle, but also the pattern of X-inactivation in muscle and blood. Their findings suggest two different mechanisms in carriers. One occurs in carriers with a random pattern of X-inactivation, the other in those with a skewed (unfavourable) X-inactivation pattern (see Fig 11.2).

In the former group, the phenotype ranged from asymptomatic to mild weakness, and the histopathological changes were minor. Interestingly, these patients had higher dystrophin content in muscle than predicted by the number of dystrophin-positive genes. A possibility is that, in these patients, the

relatively low grade of myopathy is not sufficient to trigger a significant recruitment of satellite cells and that the compensation for dystrophin production derives from a 'biochemical' normalization (that is, by the increase of the domain of dystrophin-positive nuclei). On the contrary, patients with skewed X-inactivation showed a more severe phenotype with more significant histopathological changes and were usually classified as 'clinically manifesting carriers'; Pegoraro *et al.* found evidence of 'genetic' normalization in this group of carriers with significantly higher numbers of dystrophin-positive nuclei in muscle, compared to leucocytes (in which there is no selective pressure). Unexpectedly, however, in the skewed inactivated carriers, dystrophin was not always produced by genetically dystrophin-positive myonuclei, a production failure possibly due to the unfavourable muscle environment. A combination of the 'genetic' and 'biochemical' normalizations probably plays a different role at different ages in differently skewed carriers, therefore explaining not only the diversity of their initial presentation, but also of their long-term outcome. From a practical point of view, a muscle biopsy is now only very rarely indicated and only if DNA analysis in the affected DMD patient in the family is not available, for example, in the case of a sister or a maternal aunt of a patient with DMD who died before DNA analysis was available. Whilst DNA screening analysis can detect deletions or duplications, if this is unrevealing, there will be the concern that the carrier might be harbouring a mutation not identified by these currently available methods. This occurs in ~30% of cases. A muscle biopsy might help to assign the carrier status in this scenario, although its limitations in identifying carriers must be borne in mind.

The consensus would seem to be that convincing and clear-cut muscle dystrophin abnormalities are infrequent in healthy carriers with a normal SCK level. Therefore, such studies, which are both invasive and expensive, would seem, at least at present, to offer little additional information for carrier detection and genetic counselling.

Molecular genetics studies

There have been many attempts to improve the discriminatory value of the SCK test by combining the results with those of other tests such as muscle pathology and dystrophin expression studies. However, it is now clear that the most useful information is obtained by the direct study of the intragenic mutation, if this is known, or by combining SCK data with information from linked DNA markers when information on a possible mutation is not available. It is incorrect to consider the value of the SCK test purely in terms of the 'detection rate': It is much preferable to consider the probability (odds) of a woman being a carrier on the

basis of not only her SCK level, but also the pedigree and DNA data. It usually then makes little difference to the final probability estimate in practical terms, whether the test result is actually outside the normal range or lies in the upper part of the range.

Finally, the diagnosis of the affected male and the identification of a carrier female in a family have to be seen as an overall problem, data on the former helping to establish the status of the latter (see Fig. 11.3).

Calculation of risks

The estimation of genetic risks is usually based on Bayes' theorem (Emery 1986). In these calculations, four probabilities are considered: prior, conditional, joint, and posterior. The prior probability is based on knowledge of the individual's antecedents and sibs. The conditional probability is the probability of being a carrier or not, depending on the individual's SCK level, data from DNA markers, and the number of normal sons she may have had. The product of the prior and conditional probabilities is the joint probability. The final posterior probability of a woman being a carrier is the joint probability of getting the observed information, given she is a carrier, divided by the sum of this probability plus the joint probability of getting the observed information if she is not a carrier. The method of calculation is illustrated in the following examples.

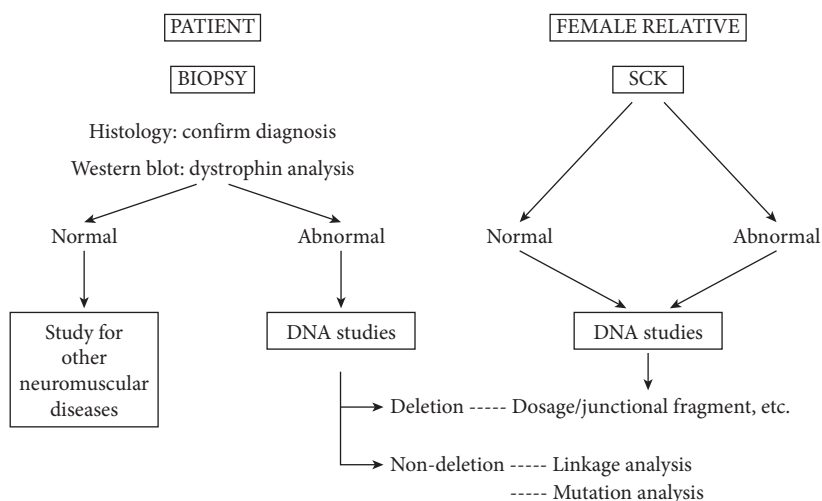


Fig. 11.3 The diagnosis of the affected male and the identification of the carrier female in a family with an Xp21 disorder.

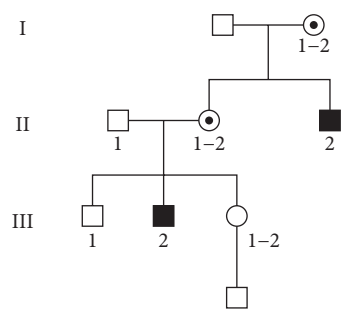


Fig. 11.4 Pedigree of DMD linked to an RFLP, the alleles of which are represented below the pedigree symbols.

Consider the family in Fig. 11.4 where a daughter III₃ with a normal son seeks genetic counselling. It would appear that the Duchenne gene and RFLP allele-2 are co-inherited in the family. First, we consider the prior probability of III₃ being a carrier or not being a carrier which is 0.5. Let us assume that she has an SCK of 40 IU, that she has inherited RFLP allele-2 from her mother, and that the frequency of recombination (θ) between the RFLP and the disease locus is 5% (0.05). Then, if she is a carrier, the (conditional) probability of her having allele-2 is 0.95, that is, 1 minus θ , because crossing-over would not have to occur. Since 56% of controls and 12% of definite carriers have an SCK of 31–50 IU (see Table 11.3), the conditional probability of having an SCK of 40 IU, if she is a carrier, is 0.12.

Finally, the conditional probability of having a normal son, if she is a carrier, is 0.50. On the other hand, if she is not a carrier, then these conditional probabilities are, respectively, 0.05 (since crossing-over would now have to occur), 0.56, and 1.00. The calculations are set out as follows.

Probability	Carrier	Not a carrier
Prior	0.50	0.50
Conditional		
Allele-2	0.95	0.05
SCK 40 IU	0.12	0.56
Normal son	0.50	1.00
Joint	0.029	0.014

Her posterior probability of being a carrier is then:

$$0.029 / (0.029 + 0.014) = 0.674$$

That is, there is a very high probability (67%) that she is a carrier, and therefore any son she has would have 1 in 3 chances of being affected.

However, suppose she had inherited allele-1, and therefore seemed unlikely to be a carrier (unless crossing-over occurred), yet her SCK level was 100 IU (that is, in the upper part of the normal range). The calculations are then as follows.

Probability	Carrier	Not a carrier
Prior	0.50	0.50
Conditional		
Allele-1	0.05	0.95
SCK 100 IU	0.144	0.015
Normal son	0.50	1.00
Joint	0.002	0.007

Her posterior probability of being a carrier is then:

$$0.002 / (0.002 + 0.007) = 0.222$$

Thus, the chance of being a carrier remains high, and, in this case, the probability of a son being affected is roughly 1 in 9.

It should be noted that, in these calculations, for the sake of simplicity, the linkage phase in the mother (whether allele-2 is co-inherited with the Duchenne gene) is assumed, and, with a closely linked probe ($\theta < 0.10$), this makes no practical difference to the results. However, nowadays, in many families, there is only one affected boy. The affected boy in such a family may represent a new mutation, and there is also no certainty as to the linkage phase in the family. Let us first consider, for the sake of simplicity, that, in such a family, only data on SCK levels are available. Let us assume that a woman who seeks genetic counselling has an SCK of 80 IU, one normal brother, and a sister with an SCK of 60 IU who has an affected son, there being no one else affected in the family. We first have to go back one generation and consider the mother of these two sisters. Like any woman in the population, she has a prior probability of being a carrier of 4μ (where μ is the mutation rate in both males and females). The reason, put simply, is that the chance of a mutation occurring in either of her maternally or paternally derived X chromosomes is 2μ , and the probability that she might have inherited the mutant gene through her mother is also 2μ . We then consider the conditional probabilities, first, of her having had a normal son and, second, of having had a daughter with an SCK of 60 IU and an affected son. In the case of the daughter, we first determine the prior probabilities of her being a carrier or not a carrier, given that her mother is or is not a carrier. Second, we

determine the conditional probabilities of the daughter having an affected son and an SCK level of 60 IU, assuming that she is or is not a carrier, and finally we determine her joint probabilities. The final overall joint probabilities are arrived at by multiplying the daughter's joint probabilities by her mother's prior probabilities and her mother's conditional probabilities of having a normal son.

The calculations are set out as follows.

Probability	Mother			
	Carrier	Not a carrier		
Prior	4μ	$1 - 4\mu \equiv 1$		
Conditional				
A normal son	$1/2$	1		
Daughter				
	Carrier	Not a carrier	Carrier	Not a carrier
Prior	$1/2$	$1/2$	2μ	1
Conditional				
Affected son	$1/2$	μ	$1/2$	μ
SCK 60 IU	0.07	0.24	0.07	0.24
Joint (daughter)	0.02	0.12μ	0.07μ	0.24μ
Joint (mother)	0.04μ	$0.24\mu^2^*$	0.07μ	0.24μ

* Negligible.

The final posterior probability of the mother being a carrier, taking into account information on her daughter with an affected son, is the sum of the joint probabilities if she is a carrier (columns 1 and 2), divided by the sum of these probabilities plus the sum of the joint probabilities if she is not a carrier (columns 3 and 4), that is:

$$0.04\mu / (0.04\mu + 0.07\mu + 0.24\mu) = 0.11$$

We now consider the sister who came for counselling, who now has a prior probability of being a carrier of 0.055, say 0.06.

Probability	Carrier	Not a carrier
Prior	0.06	0.94
Conditional		
SCK 80 IU	0.16	0.03
Joint	0.010	0.028

Her posterior probability of being a carrier is therefore:

$$0.010 / (0.010 + 0.028) = 0.26$$

Thus, despite the fact that both she and her sister have SCK levels within the normal range, the sister who requested counselling still has a high chance (namely, about 1 in 4) of being a carrier.

A general formula for calculating the probability of a woman being a carrier of a **lethal** X-linked disorder that affects either a brother or a son (**there being no one else affected in the family**) has been derived. If h_c and h_m refer, respectively, to the relative probabilities of normal homozygosity to heterozygosity (Y_1/Y_2 in Table 11.3) in the suspected carrier and her mother, so that, if there is no such information, $h = 1$, and if q is the number of normal brothers and r the number of normal sons, and if s is 1 where a son is affected and 0 if a brother is affected, and t is 0 where a son is affected and 1 if a brother is affected, then the probability (P) of her being a carrier of a **lethal** X-linked disorder is:

$$P = (1 + sa) / (1 + sa + ab + tb)$$

(where $a = h_m 2^q$ and $b = h_c 2^r$)

It is also helpful to include, in these calculations, information on SCK levels in all the first-degree post-pubertal female relatives of a suspected carrier. Over the years, one of us (Emery) has tested some 1400 potential carriers in over 400 families, in many of which there was only one affected boy. By using Bayesian statistics and combining both pedigree and SCK data, rather than using pedigree data alone, this reduced considerably the proportion of women who fell into the intermediate risk range (see Fig. 11.5). Even further separation is possible if DNA data are also included in the calculations.

Finally, even in families with an isolated case of DMD, carrier detection can be improved by using data from a linked RFLP or microsatellite polymorphic marker. The probability of the sister of an affected boy being a carrier (or a subsequent male fetus being affected) depends on whether the individual has the same or a different maternal RFLP (or other markers) from the affected boy. If the sister has a different allele, then barring crossing-over, she is unlikely to be a carrier. The position is improved, the closer the DNA marker is to the disease locus. If θ is, say, 0.05 and she has a different allele from her affected brother, her risk becomes about 1 in 16, or <1 in 30 if she also has an unaffected brother (see Fig. 11.6). The risks could be reduced even further, depending on her actual SCK level.

Information on DNA markers lying on either side of the locus (flanking or bridging markers) and from the maternal grandfather's haplotype is also

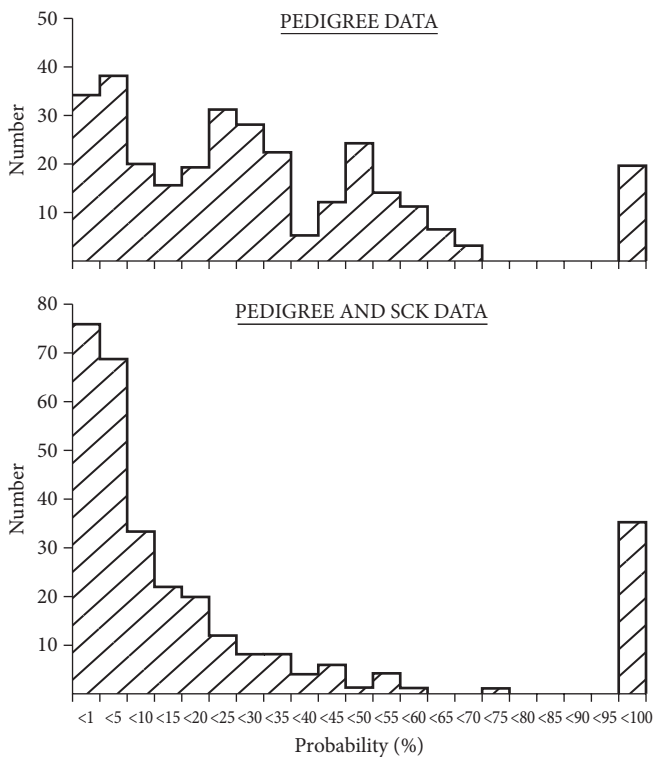


Fig. 11.5 Risks in 300 potential carriers, based on: pedigree data alone (above); pedigree and SCK data combined (below).

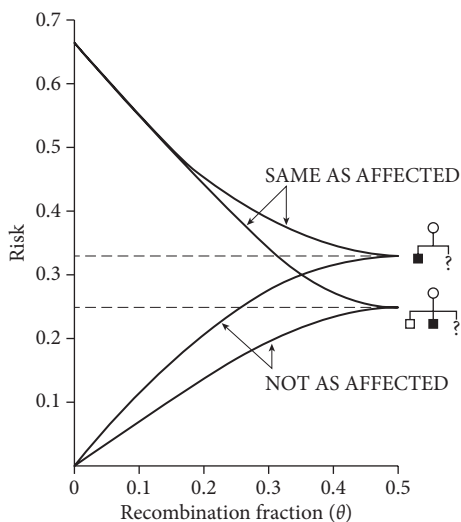


Fig. 11.6 Risks of the sister of an isolated DMD case being a carrier (or a subsequent male fetus being affected), depending on whether the individual has the same, or a different, maternal RFLP allele from the affected boy.

important and increases the precision. The likelihood that information from a linked RFLP (or other markers) will be helpful (the mother will be heterozygous, and the segregation pattern in the family will be informative) increases, as the number of alleles at the marker locus increases.

The calculations involved in determining the probability of the mother or sister of an isolated case being a carrier, which takes into account both SCK and RFLP (or other markers) data, are detailed in Emery (1986) and Young (1991). However, they can be somewhat tedious, especially when more than one DNA marker is involved and there are a number of relatives to be considered. All these calculations are now performed using computer programs. Too much reliance on such programs, however, may lead to problems, because serious errors can occur if mistakes are inadvertently made in inserting relevant data. When dealing with straightforward familial cases, and in isolated cases where one is only dealing with a single closely linked probe, the calculations can often be performed with a hand calculator.

A guide to the risks of a daughter, whose brother is an isolated case, being a carrier is given in Table 11.5. The risks depend on her SCK level (the relative probability of normal homozygosity to heterozygosity ' h '), whether she has the same or a different RFLP (or other markers) allele as her affected brother, and the frequency of recombination (crossing-over) between the RFLP and the disease locus (0.01–0.15). The risks of the mother having another affected son correspond to the entries in the table where $h = 1$ (that is, where, in a potential carrier, it is assumed there is no information on SCK levels). These risks, however, ignore additional information that might also be available, including the number of normal sons and brothers the mother may have, the mother's SCK level, information from more than one marker, and the haplotype of the maternal grandfather. Nevertheless, the tabulated risks provide at least a first approximation. Note that, until there is more information about subsequent sons and her daughter's status, the risks of the mother being a carrier are a priori 2 in 3 and obviously uninfluenced by DNA marker data on her affected son.

One further point is as follows. The carrier status of a female in an **affected family** without a deletion or duplication may be deduced from information obtained at prenatal diagnosis. If a male fetus is found not to have any dystrophin, then clearly the mother must be a carrier (or a germline mosaic). If the fetus has normal dystrophin expression, then, of course, this tells us nothing of the mother's genotype.

The particular value of linked markers is that they may provide helpful information, even in a family where an isolated affected boy is now deceased. Here, it may be possible to show, for example, that the male fetus of a sister of the

Table 11.5 Risks of the sister of an isolated DMD case being a carrier (or of a subsequent male fetus being affected) for different values of h and recombination fraction (θ), and whether the sister (or male fetus) has the same*, or a different†, RFLP allele from the affected boy (when $\theta = 0.50$, there are no data on a DNA marker; when $h = 1.0$, there are no data on SCK)

	Recombination fraction (θ)			
	0.01	0.05	0.10	0.15
$h = 0.1$				
Same	0.950	0.938	0.923	0.908
Different	0.118	0.403	0.577	0.672
$h = 0.2$				
Same	0.904	0.884	0.858	0.831
Different	0.063	0.253	0.405	0.506
$h = 0.5$				
Same	0.790	0.753	0.707	0.664
Different	0.026	0.119	0.214	0.291
$h = 1.0$				
Same	0.653	0.603	0.547	0.497
Different	0.013	0.063	0.120	0.170
$h = 2.0$				
Same	0.485	0.432	0.376	0.330
Different	0.007	0.033	0.064	0.093
$h = 3.0$				
Same	0.386	0.336	0.287	0.248
Different	0.004	0.022	0.043	0.064
$h = 4.0$				
Same	0.320	0.275	0.232	0.198
Different	0.003	0.017	0.033	0.049
$h = 5.0$				
Same	0.274	0.233	0.194	0.165
Different	0.003	0.013	0.027	0.039

$$*\text{Risk} = \left(1 + \frac{h(1 + 4\theta - 4\theta^2)}{2 - 4\theta + 4\theta^2}\right)^{-1} \quad \dagger \text{Risk} = \left(1 + \frac{h(3 - 4\theta + 4\theta^2)}{4\theta - 4\theta^2}\right)^{-1}$$

affected boy has inherited the grandpaternal X chromosome haplotype and therefore is unlikely to be affected. Linkage studies may be the only approach to carrier detection when there is not a deletion. It must be remembered that it is important to use a panel of intragenic markers so that the errors due to recombination are reduced. The occurrence of recombination in a family in which it is not known where the mutation lies prevents the status of an at-risk fetus from being unequivocally determined.

Direct carrier detection

The methods of diagnosis that depend on linkage are referred to as indirect, since they do not identify the mutation itself, but only its location with respect to DNA markers. Methods that aim to identify the mutation are referred to as direct. These latter methods should, at least in theory, make a precise diagnosis possible.

Dosage

This study can be applied to carriers of a dystrophin gene deletion or duplication. Initially, this test was performed by Southern blot analysis with appropriate cDNA probes. A dosage difference between controls and carriers, in the ratio of 2:1 for deletions and 2:3 for duplications, might be expected. Unfortunately, in most carriers, such differences are not convincing. This methodology has been superseded by quantitative PCR amplification using fluorescent primers. By using polymorphic markers or exon-specific primers located inside and outside the deleted area, a confident carrier status can be assigned. The technique requires careful standardization, but, in experienced hands, it is an extremely powerful tool for these studies in carriers.

Junction fragments

A deletion or duplication may generate a so-called 'junction fragment' of altered size if the breakpoint occurs close to a non-deleted exon so that it lies within the restriction fragment detected by a cDNA probe. This is recognized as an additional band on a Southern blot. If a deletion-associated junction fragment occurs in a family, then all affected males and all carrier females in the family will have this additional band. Unfortunately, with Southern blot analysis and cDNA probes, at most, only 20% of deletions are associated with an identifiable junction fragment. However, by using field inversion gel electrophoresis (FIGE) or pulsed-field gel electrophoresis (PFGE), the proportion of cases in which a junction fragment is seen can be increased to over 90%. These techniques are technically very demanding and time-consuming and require expensive equipment. They have been currently almost entirely superseded by the quantitative PCR approach (see Fig. 11.7).

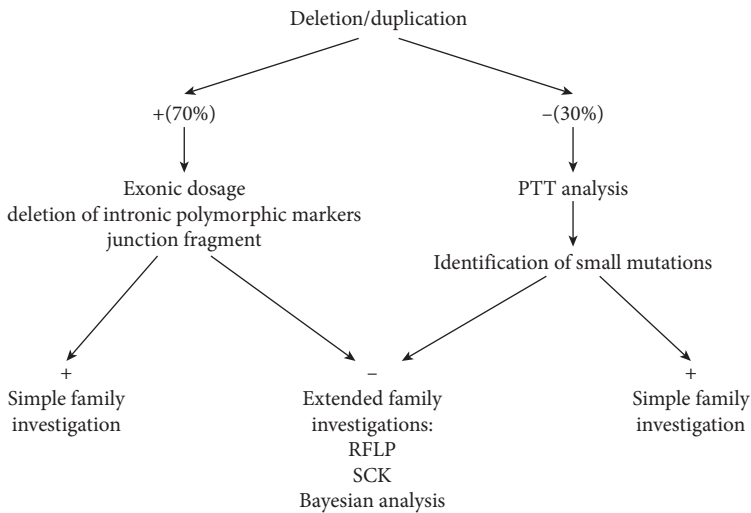


Fig. 11.7 Investigation of families with or without a gene deletion or duplication (unless a point mutation can be detected directly, for example, by a single-strand conformation polymorphism).

RNA studies (protein truncation test)

A novel approach to carrier detection was developed by Roberts and colleagues in the early 1990s (Roberts *et al.* 1990, 1991). Initially developed for amplifying (by ‘nested’ PCR) reversely transcribed mRNA from peripheral blood lymphocytes in which dystrophin mRNA is ‘illegitimately’ transcribed because of technical difficulties, it has more recently been confined to muscle RNA. Amplified RNA is translated into protein using an *in vitro* system, and the proteins are run on a gel. If a fragment carries a mutation (out-of-frame deletion or non-sense point mutation, for example), the resulting protein product will be truncated—hence the name of the method protein truncation test (PTT). The cDNA corresponding to the truncated fragment is then sequenced, and the mutation identified. Again, these tests are now only very rarely used, as genomic techniques can provide a final diagnosis in most instances.

Automated DNA analysis

Approaches that are currently being used in some laboratories are automated direct sequencing of the entire dystrophin gene, and the application of DNA microarray technology (Hegde *et al.* 2008) and next-generation sequencing (Lim *et al.* 2011). Both techniques detect the great majority of mutations but are not available on a large scale. Best practice guidelines for the genetic diagnosis of DMD, BMD, and carriers are available (Abbs *et al.* 2010).

Table 11.6 Summary of approaches to carrier detection in families with DMD*

	Available		Unavailable
Affected male			
Deletion/duplication	+	–	
	(70%)	(30%)	
Possible carrier			
SCK	+	+	+
Linkage studies	+	+	(+)
DNA studies			
Dosage	+	–	(+)
Junction fragment	+	–	(+)
<i>In situ</i> hybridization	+	–	(+)
Lymphocyte RNA	+	–	(+)
DNA analysis	+	+	+
Muscle dystrophin	(+)	(+)	(+)

* +, useful; (+), possible; –, not indicated.

In conclusion, in familial cases where DNA samples from affected individuals are available and if a gene deletion or duplication is present, then carrier identification (and prenatal diagnosis) is usually straightforward (see Table 11.6). Even when there is only one affected male in the family, but DNA is available, then carrier identification is often possible using these methods. A problem arises when the only affected member of the family is now deceased, although, with the recent introduction of reliable methods of quantitative PCR, it is possible to identify deletions and duplications also in carriers, not only in affected boys. The various approaches available for carrier detection are summarized in Table 11.6.

Prenatal diagnosis

A woman at high risk of having an affected son may choose fetal sexing with selective abortion of any male fetus and, in this way, be guaranteed a daughter who will not be affected. This is, however, almost invariably not necessary nowadays, as a reliable test for the affected male fetus is possible in most families in which DNA from the propositus is available. Fetal DNA can be extracted using either amniotic fluid cells, obtained by transabdominal amniocentesis at about 16–18 weeks of gestation, or more recently chorion

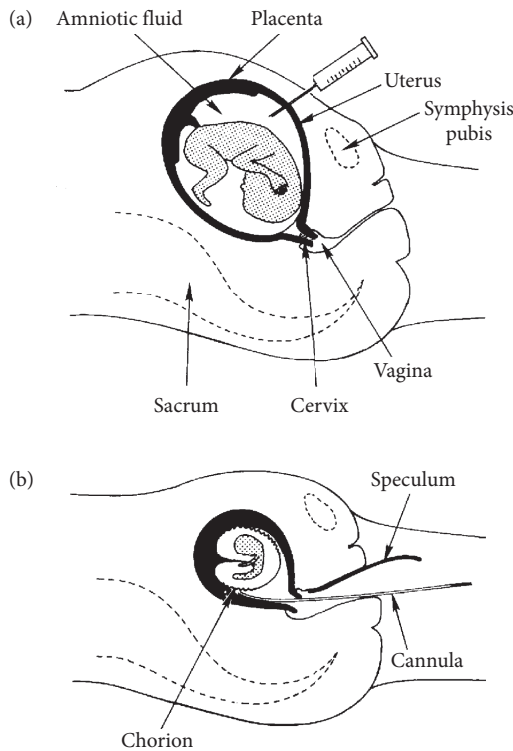


Fig. 11.8 Technique of: (a) transabdominal amniocentesis; (b) chorion biopsy.

Reproduced from Emery, A. E. H. and Malcolm, S., *An introduction to recombinant DNA in medicine*, second edition, Wiley, Chichester, UK, Copyright © 1995, with permission from John Wiley and Sons Ltd.

biopsy. Essentially, this latter technique consists of inserting a flexible cannula/catheter, either through the cervix or transabdominally into the uterine cavity. Chorionic villi (which are of fetal origin) are carefully removed for DNA, cytogenetic, and other studies (see Fig. 11.8). Since this procedure can be performed as early as 10 weeks' gestation and the material need not be cultured for DNA or chromosome studies, a prenatal diagnosis can be made much earlier than with amniocentesis, and, if an abortion has to be carried out, it is therefore likely to cause less psychological trauma.

DNA studies on cultured amniotic fluid cells or chorionic biopsy material can establish a fetal diagnosis on the basis of linkage studies when there is no deletion or duplication, or by demonstrating a rearrangement at Xp21 in other cases.

Fetal muscle biopsy

Fetal muscle biopsy has also been employed in the past for the diagnosis of an affected fetus. The technique involves inserting a trocar and cannula through the myometrium into the amniotic cavity under ultrasonographic guidance in the second trimester of pregnancy. Diagnosis is then based on histological and dystrophin immunohistochemical studies on the biopsied material, and, remarkably at birth, there is little more than a small scar in the biopsy region. The technique is, however, difficult and the possibility of fetal loss can be high.

Fetal muscle dystrophin

In the normal embryo, dystrophin first appears in the sarcolemma at the peripheral ends of the myotubes, immediately adjacent to the tendons. In the fetus, it appears throughout the entire myofibre, becoming restricted to the sarcolemma only later.

Examination of muscle tissue from fetuses affected with DMD and aborted in the second trimester of pregnancy has revealed a complete absence of dystrophin in some fetuses, as well as an increased variation in fibre size and an increased number of hypercontracted fibres, with an increase in intracellular calcium, which confirms that these histological changes are, in fact, an early manifestation of the dystrophic process. The possibility of detecting truncated forms of dystrophin in a DMD fetus has also been reported, highlighting the importance of using a panel of anti-dystrophin antibodies, as discussed in Chapter 4. These immunohistochemical studies could be diagnostically important where no mutation is detectable at the DNA level.

Furthermore, such studies can be very important in helping to establish the carrier status of a mother when DNA is either unavailable from other affected relatives or is uninformative. The demonstration of a significant defect in fetal muscle dystrophin would confirm that a mother at risk is a carrier. In other cases, where haplotype information is available in an affected relative, fetal muscle studies may, in conjunction with haplotype data, prove that a mother is not a carrier. This is clearly shown in the evolution over several years in the management of a family in which the only affected male is deceased (see Fig. 11.9).

Germline mosaicism

Several authors have reported families with intragenic deletions of the Xp21 locus that were transmitted to more than one offspring by women who showed no evidence of the mutation in their own somatic (leucocyte) cells. An example of this is shown in Fig. 11.10. These findings have been attributed to germline,

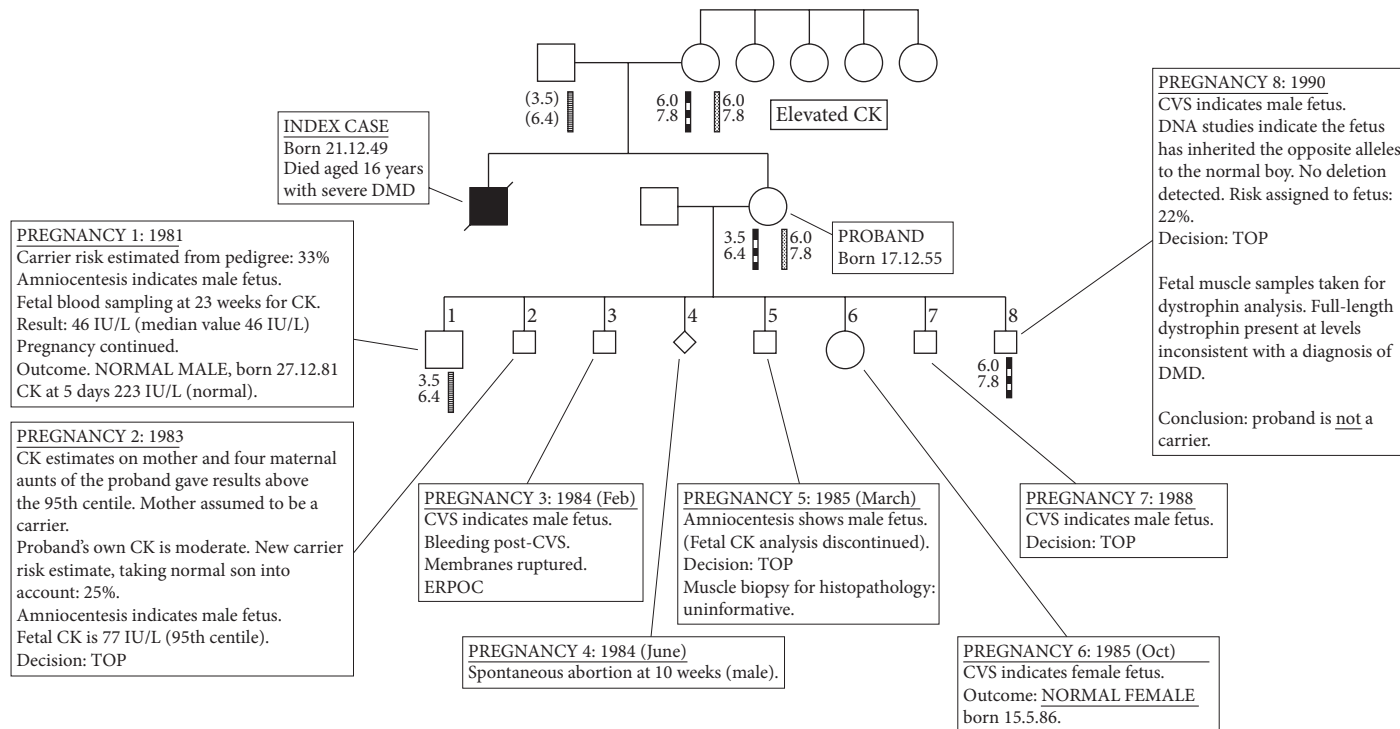


Fig. 11.9 The evolution over several years in the management of a family in which the only affected male was deceased.

Reproduced courtesy of Drs David E. Barton and Clare Davison.

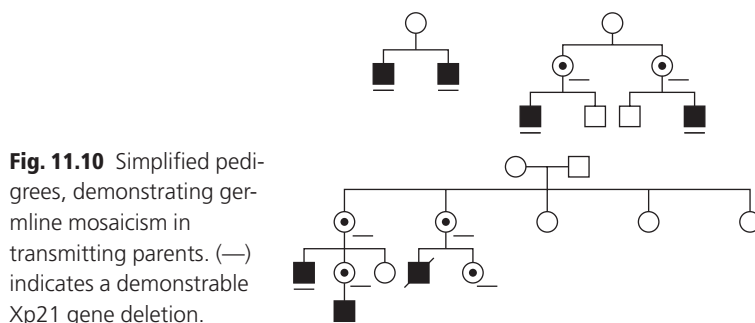


Fig. 11.10 Simplified pedigrees, demonstrating germline mosaicism in transmitting parents. (—) indicates a demonstrable Xp21 gene deletion.

germinal, or gonadal mosaicism. That is, individuals who are phenotypically normal and not genetic carriers nevertheless transmit a mutation to more than one offspring, because they harbour a somatic mutation within a fraction of their germline cells.

The phenomenon is not restricted to DMD but has also been reported in a variety of other genetic disorders. Though there is, as yet, no **direct** evidence of germline mosaicism in the case of DMD, in dominant lethal osteogenesis imperfecta, the causative mutation was detected in one in eight sperms of a normal father who had had two affected infants. In this case, the transmitting father's germline mosaicism was a reflection of generalized somatic mosaicism. A similar phenomenon was described in a family with DMD in which a transmitting male with mild muscle weakness appears to have been both a somatic and germline mosaic.

Estimates of the frequency of germline mosaicism among families with DMD have been variously calculated to be between 12 and 20%. Formulae for incorporating germline mosaicism into calculations of genetic risk have been proposed. However, from a practical point of view, it means that it can never be assumed that a male fetus, born of a mother with a normal genotype, will, in fact, be unaffected. It is therefore advisable to consider prenatal diagnosis in all pregnancies in at least the mother and sisters of an isolated affected boy. In the case of a mother without a deletion who has an affected son, it is important to test with cDNA probes all her daughters to determine if she may be a germline mosaic.

Ova transfer and preimplantation diagnosis

The transfer of ova might be indicated in the case of a woman who is at high risk of having an affected son but, who for various reasons, may not be able to face prenatal diagnosis and abortion. Ova from an unrelated (non-carrier) female may be fertilized *in vitro* by the carrier's husband's sperm. A fertilized ovum is then implanted in the carrier's uterus where it develops normally.

Another possibility is to remove ova by laparoscopy from a carrier female and, having fertilized them with her husband's sperm, allow them to develop *in vitro* until, say, the early blastocyst stage. A single cell is then removed without damaging the conceptus, and, by using appropriate DNA technology, it is determined if it will be an affected male. Only unaffected male conceptuses would be reimplanted in the uterus to undergo further development.

An even more intriguing possibility is to remove an ovum and its associated polar body prior to fertilization and, by PCR, amplify the relevant Xp21 sequence in DNA from the polar body. Any X chromosomal defect detectable in the polar body cannot be present in the ovum, which could then be fertilized *in vitro* and returned to the uterus to undergo further development.

All these techniques are feasible, although they are technically very demanding. Few preimplantation diagnoses for DMD have so far been reported.

Summary and conclusions

Since DMD is a serious disorder for which, at present, there is no effective treatment, much emphasis has been given to prevention. This involves the ascertainment of women likely to have an affected son and the provision of genetic counselling and prenatal diagnosis for such women. The ascertainment of women at risk could be achieved by screening the entire population for affected boys or by screening women within known affected families. Screening for affected boys in the newborn period has the advantage that such early detection might lead to the prevention of second cases in a family. A number of neonatal screening programmes have been developed with some success.

The major problem in prevention is the detection of female carriers. About 5–10% have some degree of muscle involvement, but this is rarely serious. A simple test for detecting healthy carriers is the determination of the SCK level. However, today, DNA-based techniques are the most reliable means for assigning carrier risk and for prenatal diagnosis. These methods can be divided into indirect methods (linkage analysis) and direct methods that depend upon identifying the mutation itself and include DNA dosage, the detection of junction fragments, PTT analysis, and DNA sequencing. Muscle dystrophin studies are only rarely indicated. The extension of DNA studies to the fetus has made prenatal diagnosis possible, either through amniocentesis in the second trimester of pregnancy or, more recently, chorion biopsy in the first trimester of pregnancy. This has also made possible the study of affected fetal muscle, which is providing novel insights into the early stages of the dystrophic process.

Finally, because of the possibility of germline mosaicism, it is advisable to consider prenatal diagnosis in all pregnancies in at least the mother and sisters of an isolated affected boy.

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Genetic counselling

Introduction to genetic counselling

Much emphasis has so far been placed on the probability of a woman being a carrier and the risks of her having an affected son. In genetic counselling, these are important issues, but other matters also have to be considered and discussed.

Genetic counselling is essentially a process of communication between the counsellor and those who seek counselling. Information to be communicated falls roughly into two main areas. First, information about the nature of the disorder: its severity and prognosis, and whether or not there is any effective therapy, reinforcing the discussion that, in most instances, will have already occurred with the paediatrician or paediatric neurologist, and also what the genetic mechanism is that caused the disease, and what the risks of its occurrence are in relatives. Second, information on the available options open to a couple who are found to be at risk of transmitting the disease. The latter may include discussions of contraception, sterilization, prenatal diagnosis, preimplantation diagnosis, and abortion.

When discussing the disease, the genetic counsellor has to present an accurate picture, even if it is depressing and disturbing, if the parents are to make a reasoned decision about future children. Such discussions require considerable sensitivity and tact, when the parents already have a young affected child and have just begun the journey of living with DMD. It is not uncommon for those involved in both the management of the disease and in counselling to find themselves in the dilemma of having to maintain an optimistic outlook for the affected child, whilst emphasizing the seriousness of the disorder when discussing its possible recurrence in any future children.

Having discussed at length the more medical aspects of the disease, the counsellor then proceeds to explain the genetic mechanism that caused it and the risks of recurrence in terms that are understandable to the individual couple. A preoccupation with risk figures can often be confusing and is best avoided. Often, couples merely want to know if there is any chance at all that it could occur again. In many cases, genetic mechanisms and recurrence risks need to be discussed only in broad terms. In any event, the actual interpretation of risks

is very subjective—what might be an acceptable risk to one couple may be quite unacceptable to another. Nevertheless, risks form a useful basis for further discussions and can be a significant factor in influencing decision making. One important fact that may have to be explained is that, being genetic, the parents cannot hold themselves in any way responsible for the disease, and every effort should be made to dispel feelings of guilt and recrimination that they may be harbouring.

If the risks are considered to be unacceptably high, the options available include family limitation, contraception, prenatal diagnosis, preimplantation diagnosis, abortion, and other techniques. Contraception, in this context, requires expert advice, because the results of failure will be far more devastating than when it is practised for purely social and economic reasons. A deep fear of having an affected child may well generate serious psychosexual problems, and, for this reason, some definitive form of contraception may well have to be considered such as tubal ligation or vasectomy. The effects of sterilization, when performed on healthy women whose families are complete, are likely to be entirely beneficial. But, in a young woman in a family with DMD, who either has no children or perhaps only the one affected child, it may have significant psychological sequelae. Counselling is especially important in these cases. To some couples, sexual abstinence may be the only acceptable alternative.

Prenatal diagnosis has added a whole new dimension to genetic counselling, and, when the result is negative, the reassurance it gives is entirely beneficial. But therapeutic abortion can cause considerable psychological trauma in many women, and, in those genetic disorders where prenatal diagnosis is possible, after termination of pregnancy following the diagnosis of an affected fetus, a significant proportion of mothers decline to undergo the procedure again. Sensitive counselling is therefore essential, both at the time of prenatal diagnosis and during the period following a therapeutic abortion. Although significant psychological trauma may be an unavoidable consequence of selective abortion, the alternative birth of an affected child is usually accompanied by even more intense feelings of guilt and depression. It is hoped that the more widely application of preimplantation diagnosis, now used in more centres, will help families to avoid the trauma of a therapeutic abortion whenever possible.

As a prelude to genetic counselling, it is important to define a couple's educational and social background, their religious attitudes, and, if possible, something of their marital relationship if information is to be presented most effectively and sensitively. The counsellor may sense that, for various ethical and other reasons, contraception, sterilization, or prenatal diagnosis are unacceptable to a couple. These matters should then not be discussed further. The genetic counsellor's role is to inform and guide, but not to coerce or impose his or her own views.

The various factors that influence the reproductive decisions after genetic counselling are complex, variable, and personal.

Non-directive counselling

The genetic counsellor's role, until relatively recently, was often seen purely in medical and scientific terms—to establish a precise genetic diagnosis and to communicate factual information about the disease and its genetics. However, more emphasis is now being given to an appreciation of the psychological aspects of counselling, a change from what Kessler in 1979 referred to as 'content-oriented to person-oriented' counselling. This change has been brought about by several factors. First, a disabling genetic disorder, such as DMD, often has profound psychological effects on the immediate family. Second, these effects may have long-term consequences and frequently extend to other relatives. Third, it has been found that couples sometimes opt for a course of action that may well be at variance with what the counsellor might have regarded as 'reasonable'. For example, in a prospective follow-up study, some years ago, carried out by one of us (Emery), of 200 consecutive couples seen in a genetic counselling clinic with various genetic disorders, a proportion of those who were told that they were at risk of having an affected child were undeterred and actually planned further pregnancies. At first sight, such behaviour might seem irresponsible, but, on careful questioning, in almost all cases, the reasons for planning further children were often very understandable, when considered from the parents' point of view. In some cases, further pregnancies were planned, because, after seeing the effects of a disorder in a previous child or in one of the parents, it was not considered sufficiently serious (congenital cataract, congenital deafness, peroneal muscular atrophy) or prenatal diagnosis was available (Sandhoff's disease, X-linked mental retardation). In other cases, the parents planned further pregnancies, because, if a subsequent child were affected, it would not survive (renal agenesis) or, if it survived, it would succumb within a year or so (Werdnig-Hoffmann disease). There was a small, but lamentable, group of couples who had no living children and dearly wanted a family at whatever cost.

Thus, a course of action that might seem irresponsible to one person may seem eminently reasonable to another. The choice should be the individual's prerogative, always provided that it is made in the full knowledge of all the facts and possible consequences. Since the genetic counsellor's role is to help couples arrive at decisions that are the best ones for themselves, genetic counselling should never be directive. Nevertheless, because DMD is such a serious and distressing condition, most counsellors may hope that couples at risk will exercise caution.

Timing of counselling—the coping process

For really successful counselling, it is essential to recognize the problems of attempting to communicate information of a personal and delicate nature in a situation when the parents may not yet have recovered from the shock of the diagnosis. They may well be harbouring feelings of guilt, recrimination, and lowered self-esteem. They may be angry and tense or just numbed by the situation. But all will be under considerable stress. The psychological sequence of events that follows the initial diagnosis is referred to as the ‘coping process’ and is similar in other stressful situations such as bereavement. Parents with a child with DMD have to face two major stressful events: at the time the diagnosis is first made, and later when the affected boy dies. On both these occasions, the family will require considerable support from all those concerned—paediatricians, physicians, geneticists, genetic associates, social workers, and nurses. It should also be remembered that the father may be just as affected as the mother, but, since men often do not express their emotions readily, this may be underestimated or even go unrecognized.

Five sequential stages have been recognized in the coping process (Emery and Pullen 1984):

- ◆ shock and denial;
- ◆ anxiety;
- ◆ anger and guilt;
- ◆ depression;
- ◆ psychological homeostasis.

The duration of each stage varies from individual to individual. Very rarely, a parent may never progress beyond the stage of denial, whilst a few may reach the stage of depression and remain at this stage. The genetic counsellor has to recognize the existence of these stages and to tailor his or her counselling accordingly. He/she has to appreciate that the assimilation of information and the process of decision making will be very much influenced by the stage in the coping process that a parent has reached.

At the very beginning, the parent may be unable to accept that the child is affected, and, at this stage, sympathy and compassion are required, until acceptance occurs. Anxiety impairs judgement and reason, and, at this stage, the counsellor should provide support and encourage the sharing of emotions. Information may have to be repeated on a number of occasions if it is to be fully understood and appreciated. The most difficult stage for the counsellor is when the parent is angry and resentful. Hostility may well be directed towards the counsellor. This has to be accepted as being part of the coping process and not

be taken personally. Gentle persuasion is indicated, although sometimes it may be necessary to withdraw temporarily and make arrangements for a later appointment when the parent's hostility and resentment may have been tempered. At the stage of depression, the effects may be such as to necessitate some form of antidepressant therapy, but it is probably at this stage that genetic counselling can begin more earnestly. Counselling should not be postponed, until homeostasis has been reached, although obviously information will be better received and understood and decisions will be more rational at this last stage.

Genetic counselling is part of the general counselling that parents with an affected child are given, and it calls for special knowledge and skills on the part of the counsellor.

Who should be offered genetic counselling?

Geneticists tend to consider risks greater than one in ten as being 'high' and risks less than one in 20 as being 'low'. This is based on early studies that tended to show that, in general, couples are more likely to be deterred from planning a pregnancy when the risk is greater than one in ten, but less so if it is less than one in 20. However, it is difficult to extrapolate from responses to genetic disorders in general to one disease in particular, such as DMD, because the so-called 'burden' of a disorder has to be included in the equation. By this is meant the psychological and, to a lesser extent, social and economic problems attendant on having a child with a serious genetic disorder. In some disorders, such as Werdnig-Hoffman disease, although the burden is great, it is of limited duration, and therefore possibly more acceptable than in DMD where the affected child survives for many years, becoming progressively incapacitated. There is good evidence that couples are often more influenced by the burden of a disease than by the actual risks of recurrence. Thus, concern among relatives about the disorder occurring in their children is only partly a reflection of their risk. It is also tempered by their individual views of the 'burden' of the disease.

In part, for logistical reasons, it has sometimes been suggested that genetic counselling in DMD might be restricted to those women whose a priori risk is greater than one in ten. But this does not seem entirely justified, because affected boys have sometimes been born to mothers whose risk had been estimated to be less than one in 20. There would seem every reason to offer counselling, where appropriate, to all first- and second-degree female relatives of affected boys, as well as to any other relative who may be anxious.

Another important issue relates to when to study at-risk female children and teenagers; a more recent complication relates to the outcome of genetic testing in at-risk pregnancies when the fetus is a female.

Regarding the first point, there is general acceptance that female children should not be screened for the condition before they reach reproductive age, as there is no benefit for these girls to know if they are carriers or not. Typically, genetic counselling is offered to females when they enter adult life; the lower age limit at which the test is offered nevertheless can vary, depending on the sexual maturity and independence of these adolescents, and the test can certainly occur before the transition to adult services, if directly requested and motivated by these adolescent females.

A more recent conundrum was highlighted by the literature regarding the long-term outcome of a prenatal diagnosis programme in the Netherlands. Current policy, widely used in the genetic community, suggests that female fetuses are not tested for carrier status. These females remain untested as adults and risk having affected offspring, as well as progressive cardiac disease. Indeed, this was the finding from this long-term study, which pushes the boundaries of current practice and recommends that the testing of females for carrier status, if requested, should be done prenatally, if fetal DNA is available, or post-natally even before adulthood (Helderman-van den Enden *et al.* 2013).

Effects of genetic counselling

The effects of genetic counselling and prenatal diagnosis in DMD can be assessed in various ways—in relation to changes in the incidence of the disorder in a community, the reproductive behaviour of those counselled, and the social and psychological effects on the family.

The effects on population incidence have already been discussed where it was concluded that, at best, this could be reduced to the occurrence of new mutations, which, in the past, represented about one-third of cases.

The effects on the reproductive behaviour of individual women who had been counselled in regard to DMD were assessed in several studies in the 1970s and 1980s. In general, those who were at high risk were often deterred after counselling and either avoided pregnancy altogether or opted for prenatal diagnosis in any future pregnancy. In Brazil, a high proportion of those at low risk were also deterred, but this may reflect the use of the information by women to gain priority help from family planning centres.

These follow-up studies also confirmed that the proportion of affected boys among births to mothers considered to be at high risk was, as expected, greater than among mothers considered to be at low risk.

However, all these studies were carried out before accurate carrier detection and prenatal diagnosis became possible using direct mutation detection, now widely

available, at least for deletion and duplication. Now that the element of uncertainty has been removed, it would be interesting to compare these early findings with studies carried out today to assess the effects of counselling and changes in population incidence of the disorder. As discussed in earlier chapters, recent figures on the incidence of DMD in populations in which neonatal screening programmes are under way appear to suggest a reduction of incidence of DMD.

The social and psychological effects of genetic counselling in DMD are much more difficult to assess. A common complaint from parents is that, at the time of diagnosis, they were experiencing considerable stress, making it difficult to accept information at all. In one extensive study in the UK, Firth (1983) reported the results of interviews with 53 families. In only 18 were both parents told of the diagnosis together. Many of the parents who had been alone when told described how their distress was heightened by having to break the news to their spouse. A third of the parents were not satisfied with the way the information had been conveyed, which was often inadequate and with no follow-up. Although conveying information about a diagnosis is only part of counselling, it is an important part. It is difficult to see how if, at this stage, a good rapport has not been established with a couple, any meaningful dialogue can follow later. On the basis of her findings, Firth made some recommendations—parents should be told of the diagnosis as soon as possible, together and in private, and a series of contacts should be planned not only with the paediatrician, but with other health care professionals involved with the disease who can provide long-term support for the family. Since her report appeared in 1983, there have been many changes. More help and guidance is now being given to families, but there is always room for improvement. Some years ago, a Working Party of the National Association for Mental Health concluded:

... telling the parents is only a first step in the continuing management of the handicapped child. It is not an end in itself and unless it leads correctly on to the appropriate involvement of other professional workers it would largely have failed in its primary object of securing for the handicapped child the fullest possible developmental goals and an accepted place in the family. (Carr and Oppé 1971)

Although these sentiments were expressed in regard to handicap in general, they are also relevant to DMD. Establishing the diagnosis and proffering genetic counselling should only be the beginning of the health professionals' involvement with the parents and the affected child. Their continuing support may well be required for several years to come.

A very readable series of reviews of the great variety of psychosocial problems associated with DMD and other neuromuscular disorders is provided by Charash *et al.* (1991). This and Firth's (1983) report should be carefully considered by anyone becoming involved in counselling families with DMD.

Summary and conclusions

Genetic counselling is essentially a process of communication between the counsellor and those who seek counselling. The information to be communicated concerns, first, the disease itself, the genetic mechanism that caused it, and the risks of recurrence, and, second, the options available if the risks are considered unacceptably high. These include contraception, sterilization, prenatal diagnosis, preimplantation diagnosis, and abortion, each of which may, in itself, have important psychological sequelae and require counselling. Some couples, for various ethical and other reasons, may be unable to accept these options. This is their prerogative, for genetic counselling should not be directive but help couples reach a decision that is the best one for themselves.

It is particularly important to recognize the psychological aspects of genetic counselling and the sequence of events that follows the initial diagnosis, and that is referred to as the 'coping process'. This involves five sequential stages: shock and denial, anxiety, anger and guilt, depression, and finally psychological homeostasis. Each stage requires counselling to be tailored accordingly, for only in this way will it be at all effective and will rational decisions be made.

Concern about the disorder occurring in various relatives is tempered by considerations of the 'burden' of the disease, as well as the individual's risks, and counselling should be offered to all those female relatives who are anxious about the problem.

The effects of genetic counselling can be assessed in several ways. There are indications that the population incidence is being reduced to the occurrence of new mutations. Familial cases are now becoming less common. Studies of the reproductive behaviour of individual women who have been counselled indicate that those at high risk are very largely deterred from pregnancy, unless coupled with prenatal diagnosis. Finally, the social and psychological effects of genetic counselling can be assessed, but so far this has received little attention in the case of DMD. Indications are that there is often some dissatisfaction with the way in which the diagnosis is first made and a lack of subsequent follow-up. There is a real need to ensure that parents are told accurately and compassionately as soon as possible. Thereafter, a series of contacts can be offered and planned with various health care professionals involved with the disease. The latter can then provide, if need be, long-term support for the family as a whole.

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Management

Introduction to management of Duchenne muscular dystrophy

With the identification and characterization of the defective protein in DMD and BMD, prospects for a rational therapy are beginning to seem a reality. Therapeutic strategies for development include: agents to reduce secondary muscle damage and/or promote muscle regeneration after injury (such as IGF-1); and strategies to increase the expression of dystrophin on the sarcolemmal membrane, using either gene therapy or RNA-based approaches. However, it may take some years before any successful treatment is licensed for use in routine clinical practice. Details of ongoing clinical trials for DMD can be found on the TREAT-NMD website (<<http://www.treat-nmd.eu/>>). In the meantime, therapy involves a complex package of multidisciplinary care to prolong ambulation and proactively manage cardiac and respiratory complications. Good multidisciplinary care can vastly improve the quality of life and life expectancy. In countries where there is centralized multidisciplinary care, such as Denmark, for example, life expectancy for DMD can now reach 47 years of age (personal communication from Professor Jes Rahbeck 2013). Survival data for DMD have highlighted significant inequalities in care across different European countries, and even between regions within countries. In an attempt to overcome such inequalities, international consensus standards of care have been developed and, in the UK, are accredited by National Institute for Health and Care Excellence (NICE). The process by which these standards were derived is beyond the scope of this chapter, but the reader can download the original *The Lancet* publications from the TREAT-NMD website (Bushby *et al.* 2010a, 2010b); a lay version for families is also available in several languages. Specific aspects of multidisciplinary care are dictated by the age of the child and stage of the disease. Corticosteroids and physiotherapy are most effective in the early stages of the condition and are used to prolong independent walking. In the older child, surgery and orthoses may promote indoor walking and prolong standing, whilst, in the non-ambulant child, scoliosis management and correct seating are imperative. With advancing age, there will be greater emphasis on nutritional, gastrointestinal, respiratory, and cardiac management (although

cardiac screening should start at diagnosis). At all stages, there should be an awareness of the effects of the disease on behaviour, education, and social participation which, in turn, affect the quality of life for both the child and his family.

Corticosteroid management

Data from randomized controlled trials and non-randomized studies using corticosteroids (prednisolone, prednisone, and deflazacort) demonstrate an increase in muscle strength, with subsequent slowing of progression of the disease (Dubowitz 1997, 2000; Manzur *et al.* 2008). When children with DMD are commenced on corticosteroids, parents often report seeing a benefit within 2 weeks of starting treatment and comment on the fact that the child has more energy and may fall less frequently. This improvement continues for up to 6 months, following which there is a period of relative stability. Children treated with corticosteroids will remain independently ambulant for longer, and the incidence of scoliosis is greatly reduced (Alman *et al.* 2004). Furthermore, evidence from long-term non-randomized observational studies suggest that corticosteroids may have a cardioprotective effect (Markham *et al.* 2005; Silversides *et al.* 2003) and delay the onset of respiratory muscle weakness (McAdam *et al.* 2012a). Several corticosteroid regimens have been tried, although only daily and alternate daily regimens have been compared in randomized controlled trials. The most effective starting dose is prednisolone 0.75 mg/kg or deflazacort 0.9 mg/kg. Both are equally effective; there is perhaps less weight gain with deflazacort, but the incidence of cataracts is greatly increased, compared with prednisolone (Manzur *et al.* 2008, Ricotti *et al.* 2013). Evidence from randomized controlled trials shows that increasing the dose of prednisolone to 1.5 mg/kg/day has no additional benefit, but more side effects.

There have been no trials to determine the optimum age to start corticosteroids, but expert professional consensus from anecdotal data suggests that this should be somewhere between the ages of 4 and 6 years when the child's motor development reaches a plateau. Parents must be fully informed of the relative benefits and risks of corticosteroid treatment and given the option of choosing a daily or intermittent regimen—10 days on and 10 days off (10/10) for their son. Data from 360 DMD patients, collected over 5 years as part of a national prospective audit by the North Star Network, showed that the mean age for loss of ambulation was 12 years for boys treated 10/10, and 14.5 years for those on daily treatment. Thus, maximum benefit is obtained from daily corticosteroids; however, the side effect profile of daily treatment was shown to be much greater than 10/10 and may not be acceptable to all parents (Ricotti *et al.* 2013).



Fig. 13.1 A 17-year-old boy treated with intermittent steroids (10 days on, 10 days off). He lost ambulation just before his thirteenth birthday. At 17 years, he has good sitting posture and can raise his arms above his head.

There have been no randomized controlled trials to assess the use of corticosteroids in non-ambulant boys; however, our clinical observation at Great Ormond Street Hospital and Queen Square shows that steroid-treated non-ambulant boys have better upper limb function, sitting posture, and respiratory function than untreated patients (see Fig. 13.1). Preserved upper limb function is important for feeding, and, when DMD boys lose this ability, they may lose weight and become cachectic. On the other hand, excessive weight gain can be a very serious issue in corticosteroid-treated boys, and secondary glucose intolerance or diabetes may develop. Monitoring the urine for glycosuria is

essential at each clinic visit. The minimum therapeutic dose of corticosteroids in non-ambulant boys may be less than the dose required in ambulant patients; however, the data from trials to assess this are currently lacking.

Management of corticosteroid-induced side effects

Immune system

Because corticosteroids are known to affect the immune function, it is essential to ensure that the child is immune to chickenpox prior to starting treatment. This is assessed by a blood test to measure varicella IgG antibodies. A history of chickenpox is not sufficient to assume immunity. If the child is found not to be immune, he should be vaccinated before corticosteroid treatment is started. Likewise, if there appears to be a high risk for tuberculosis, as is the case for some communities, the child should be vaccinated before starting treatment.

Weight

Treatment with corticosteroids will induce a ravenous appetite, which peaks in the first 6 months of treatment. The child should be referred to see a dietician to help parents identify low-calorie options for meals and snacks. Close monitoring of the child's weight is essential. If the child does gain significant amounts of weight, it is extremely important to adjust the corticosteroid dose, according to the predicted weight, rather than the actual weight; otherwise, severe side effects may ensue. Once started on steroids, the dose should be adjusted, according to the child's motor skills and growth. Evidence from randomized controlled trials suggests that the minimum effective dose of prednisolone is 0.3 mg/kg/day. The maximum dose of prednisolone should be capped at 60 mg/day. In reports in which long-term daily corticosteroids have been used, rarely children received prednisolone doses above 0.5 mg/kg day, once in the teens.

Bone health

One of the major side effects of corticosteroids is osteoporosis. This is due to increased osteoclast activity, reduced osteoblast activity, increased excretion of vitamin D via the kidneys, and reduced vitamin D absorption from the intestine. Furthermore, weakened skeletal muscles and loss of mobility reduce bone 'loading', which may exacerbate osteoporosis; this is most pronounced in the wheelchair-dependent child. Osteoporosis increases the risk of traumatic fractures, which, in turn, may result in the permanent loss of independent ambulation. The risk of vertebral fragility fractures increases with cumulative corticosteroid dose and may be as high as 30%; the exact figure is unknown due to a lack of reporting, as many of these fractures are 'silent' and can only be

detected on a lateral spine X-ray (Quinlivan *et al.* 2010). Acute traumatic vertebral fragility fractures often follow a fall onto the buttocks.

Upper limb fractures usually result from falls sustained whilst the boy is in the late stages of walking and is unsteady on his feet. Lower limb fractures are more common in wheelchair-dependent boys. In a survey of the prevalence, circumstances, and outcome of fractures in DMD children attending four UK neuromuscular clinics, around 20% of the 378 patients studied had experienced fractures. Of these, ~40% of fractures were in boys aged 8–11 years. Falling was the most common cause. Upper limb fractures were most common in boys using KAFOs, whilst lower limb fractures predominated in the remaining boys. Twenty per cent of ambulant boys, and 27% of those using orthoses permanently, lost mobility as a result of the fracture. The fractures themselves, however, heal normally without complications.

International consensus guidelines for bone protection in children with DMD have been published, although it is fair to say that they are still in evolution (Quinlivan *et al.* 2010). A routine bone mineral density scan (DEXA) is recommended every 1–2 years. If the adjusted spinal bone mineral density Z score is less than –2.0, or if there is a history of acute back pain, a lateral spine X-ray should be performed to exclude vertebral fragility fractures. However, it should be noted that lumbar backache is quite common in DMD boys and is most often postural in origin, secondary to lumbar lordosis. Back pain, secondary to a vertebral fragility fracture, is often thoracic and exacerbated by movement or jolting, for example, during a car journey. If a vertebral fragility fracture is found, treatment with an intravenous (IV) bisphosphonate is recommended. The use of prophylactic oral or IV bisphosphonate treatment to prevent bone loss remains controversial and currently is not recommended. Careful attention to oral hygiene is important for any child receiving bisphosphonate treatment, because acute mandibular necrosis is a recognized serious complication. However, acute mandibular necrosis has only been described in malnourished adults with coexisting serious medical conditions; as yet, it has not been reported in children.

Serum levels of 25-OH vitamin D are frequently suboptimal in boys with DMD. This could be related to poor diet, or it might be that boys with DMD prefer not to play outside in the sunshine for fear of falling. Vitamin D is important for bone accrual in children, and observational evidence suggests that maintaining a serum 25-OH vitamin D level which is at the upper limit of normal with daily supplements may increase bone accrual and reduce the incidence of vertebral fragility fractures. Paying attention to dietary calcium is also important, especially during the pubertal growth spurt; advice from a dietician can be very helpful in ensuring an adequate dietary calcium intake.

Growth failure and pubertal delay are important side effects of corticosteroids. Stature in DMD boys is already below average (the mean height is approximately on the 25th centile), and chronic corticosteroid treatment severely affects growth, to the point that children receiving daily deflazacort were ~15 cm shorter than untreated DMD boys by the late teens. Pubertal delay may exacerbate osteoporosis, and short stature can result in bullying and emotional problems. Thus, testosterone treatment should be considered when the onset of puberty is delayed beyond 14 years of age. The evidence for use of growth hormone treatment for short stature in DMD is less convincing and remains controversial. Previous reports of children with idiopathic growth hormone deficiency and DMD showed a better prognosis due to the small size of the child. On the other hand, one observational study of the use of growth hormone to treat corticosteroid-induced growth failure did not demonstrate any major side effect (although some children developed diabetes and raised intracranial pressure) and showed an increase in growth velocity after 1 year of treatment (Rutter *et al.* 2012).

General health

Corticosteroids have a great number of other unwanted effects which require regular monitoring. Cushingoid features are common, especially when treatment is administered daily (see Fig. 13.2). The child develops a ‘moon face’, acne, and increased bodily hair, which can be distressing for some. Hypertension is a common side effect, sometimes requiring treatment, and, as already mentioned, screening for glycosuria is important. Some children are troubled by fungal infections, particularly affecting the nails and feet. Cataracts and ocular hypertension are potentially serious side effects. One of the authors (Quinlivan) has seen two children who developed glaucoma, secondary to corticosteroid use (Quinlivan *et al.* 2010)—in one child, the problem resolved by reducing the corticosteroid dose; in the other child, corticosteroids had to be discontinued, because the ocular pressures did not reduce with standard treatment. Abdominal pain due to gastric ulceration is another potentially serious side effect of corticosteroids, leading to a risk of gastric perforation. Systems enquiry at each visit should include symptoms of indigestion and abdominal discomfort. Symptoms usually respond to H2 antagonists, such as ranitidine, or proton pump inhibitors such as omeprazole; referral to a gastroenterologist for endoscopy may be necessary if symptoms do not resolve.

It is advisable that DMD boys on corticosteroid should not only carry a steroid card, but also have rapid access to their local paediatric unit when unwell, as decompensation due to adrenal suppression in these children can be very rapid with serious consequences.



Fig. 13.2 An 11-year-old boy with DMD on daily corticosteroids. He has short stature, with a growth velocity of 1–2 cm/year; however, he has no lumbar lordosis, and he can run and jump.

Mood and behaviour

Corticosteroids are well known for their ability to alter mood. In children with DMD, there may already be significant learning and behavioural problems, and the additive effect of corticosteroids can pose major difficulties for parents and schools. There is an increased incidence of ADHD in boys with DMD, which can be exacerbated by corticosteroid treatment, and, unless the schools are informed of this potential adverse effect, the child may be accused of being naughty or badly behaved and be excluded from school. Exacerbation of temper tantrums is a common complaint, and parents may require the support of a psychological specialist in the children and adolescent mental health services

(CAMHS) team to manage this. Depression can be a serious side effect of corticosteroids in some people. Discontinuation of corticosteroid treatment in boys who developed severe clinical depression is a rare, but well-known, complication which, however, resolves completely when prednisolone is discontinued.

Stopping corticosteroids

Children with DMD are prescribed corticosteroids for many years, and, as a consequence, they are potentially at risk of secondary adrenal failure. It is essential that parents are informed to keep an adequate supply of tablets at home at all times. If corticosteroid treatment is discontinued, either because of adverse effects or lack of benefit, a plan for gradual weaning should be discussed with the parents. Lack of perceived benefit is an important reason to withdraw treatment, and it is important that the patient's and parents' perception of benefit is explored at each visit, especially for non-ambulant patients.

Importantly, the risk of adrenal failure can still be present, even 6–12 months after having completely stopped corticosteroids, and families and local teams should be counselled accordingly.

It is extremely important for the clinician to be aware of the additional emotional and financial burdens that corticosteroid treatment places on the child and his family, on top of the burden of caring for a disabled child. These can be substantial and may lead to hardship, marital breakdown, and even psychological disorders in the parents.

Musculoskeletal management

Physical therapies

An excellent review of physical therapies for DMD can be found in Thompson (1999). A summary of management can be found in Box 13.1.

Active exercise

Parents often ask whether they should encourage active exercise, in the belief that this might improve muscle strength. Early studies of any possible beneficial effects of exercise in DMD are difficult to interpret for various methodological reasons. But it is clear that an overtly aggressive approach to physical activity could well be counterproductive and possibly aggravate the cardiomyopathy that is a concomitant of the disease. Furthermore, the detrimental effect of excessive physical activity is highlighted in the various animal models of muscular dystrophy. In *mdx* mice, a regular programme of exercise or activity against resistance accelerates the course of the disease and worsens the pathology.

Box 13.1 Physical management in DMD

Promotion of ambulation

- Corticosteroids
- Weight control
- Exercise and stretching: active/passive
- Night splints
- Tenotomies and orthoses for ambulation

Prevention of deformities

- Posture/support/orthoses
- Standing
- Passive exercise/stretching
- Scoliosis surgery

Therefore, exercise against resistance is not recommended in DMD. In any event, activities involving recreational sports are generally more likely to win long-term adherence. Swimming is particularly valuable, as the buoyancy of the water makes exercises easier to perform. Cycling can also be beneficial, although, quite early on, boys with DMD find this difficult. The best advice would be to encourage normal physical activities as far as they are possible. In the non-ambulant child, sporting activities are also important. Wheelchair football is very popular with DMD boys, especially teenagers; coaching and local teams are organized by 'Aspire', and there is a league table organized by the Wheelchair Football Association (WFA) (see Fig. 13.3).

Passive exercise and physiotherapy

Various studies have shown that passive stretching exercises are valuable in preventing, or at least delaying, the development of muscle contractures, which are especially likely to develop in the late stages of the ambulation phase or once the child becomes wheelchair-dependent.

The role of physiotherapy in DMD goes, however, beyond the simple prevention of skeletal deformities. Several detailed physiotherapy assessment protocols have been formulated for a comprehensive evaluation of the strength and function in DMD and to enable accurate monitoring of disease progression, timing of therapeutic interventions, and documentation of the effects of any intervention. Some have been reviewed by Manzur (2001), but, more recently, newer functional assessments have been added to the list such as the



Fig. 13.3 Wheelchair football.

Image reproduced courtesy of ASPIRE WFA.

6-minute walk test and the NSAA. Standardized functional protocols, such as the 6-minute walk test, have been used in multicentre research studies as the primary outcome measure.

The two goals of physiotherapy are the prevention of deformities and the prolongation of ambulation. The prevention of deformities is particularly important in the early phases of the disease and includes passive stretching of the Achilles tendon, knee, hip, shoulder, and, to a lesser extent, the elbow and wrist joints. These stretching exercises should be carried out daily in children with DMD and are best accomplished by the parents or support worker at school, after an induction session with a professional physiotherapist. Sylvia Hyde (1984) has produced a helpful guide to such exercises for parents to use in the home (see Fig. 13.4). A practical list of the exercises indicated at different stages of the disorder is shown in Box 13.1. The emphasis is on firmness and kindness, and the aim is to prevent contractures from developing. There is no doubt that a routine of passive exercises each day, perhaps after a nightly bath, will help prevent contractures. Despite being demanding and time-consuming, it offers one of the few opportunities where parents can feel involved in doing something for their son.

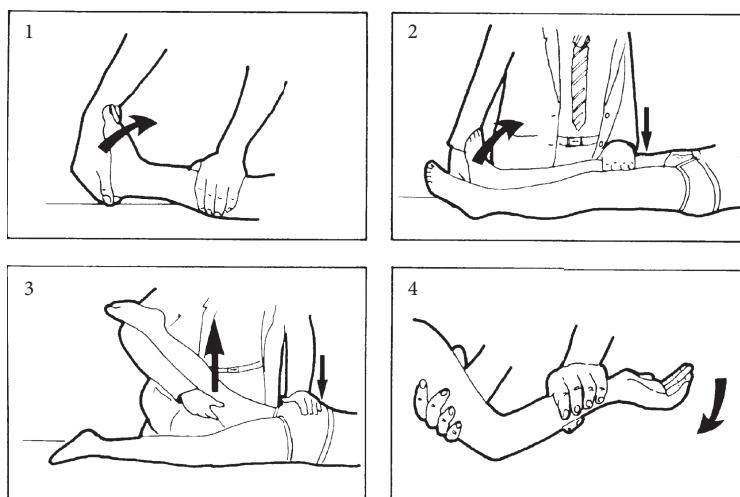


Fig. 13.4 Some of the passive stretching exercises to prevent contractures of: (1) the Tendo Achilles, (2) the knee, (3) the hip, and (4) the elbow joints.

Reproduced with permission from Hyde, S. A., *The parent's guide to the physical management of Duchenne muscular dystrophy*, Muscular Dystrophy Group of Great Britain and Northern Ireland, London, UK, Copyright © 1984.

Night splints are invaluable aids to the prevention of tightness of the Achilles tendons. They should always be prescribed, once the ankles cannot be dorsiflexed beyond the neutral position. Whilst the use of long-leg night splints, in order to help prevent the development of contractures of the ankle and knee joints, is theoretically more beneficial than using isolated ankle splints, many children find long-leg splints uncomfortable, and, in our experience, compliance is very low. The use of ankle splints is well tolerated by most children, especially if their use is introduced gradually over a period of a week whilst the child is asleep.

In a prospective study of passive stretching and splintage in boys with DMD, a delay in the loss of dorsiflexion was noted in boys whose families complied with treatment. In boys who were non-compliant, the deterioration in functional level was most marked. The effectiveness of night splints (ankle-foot orthoses) has been demonstrated in a randomized study in ambulant boys with DMD. The best results were obtained in those children who, in addition to stretching, also had night splints (Hyde *et al.* 2000). Whilst it has not been formally proved in randomized studies that these measures will prolong ambulation, it is clear, from various open studies, that early institution of physiotherapy helps in the prolongation of ambulation.

Table 13.1 Practical approach to the prevention of deformities in the ambulant stages of DMD

Intervention	Timing	Comment
TA stretching	As soon as contractures are present	Typically already at diagnosis
Night splints	If loss $\geq 20^\circ$	Commonly a few years after diagnosis
Hip stretching	When contractures detected	Common towards late phases of ambulation
ITB stretching	When contractures detected	May occur during late phases of ambulation
Knee stretching	When contractures detected	Rarely needed; may be found in children with asymmetrical ankle contractures

TA, Achilles tendon; ITB, ileotibial band.

We currently encourage children with DMD to wear ankle night splints, whenever significant (that is, $>20^\circ$) limited dorsiflexion has occurred (see Table 13.1). In our practice, the rate of compliance is very high ($>90\%$).

Early surgery

The **early** release of contractures in ambulant boys, with percutaneous Tendo Achilles lengthening, hip and knee flexion contracture release, and bilateral dissection of tensor fascia lata, otherwise known as the Rideau procedure, was previously recommended (Rideau *et al.* 1986). It was claimed that this surgery improved gait and reduced Gowers' time. However, controlled trials of **early** release of lower limb contractures, following the protocol introduced by Rideau, failed to show any benefit, and this procedure is no longer recommended.

Prolongation of walking with orthoses

Prolongation of walking in DMD can be achieved by fitting light plastic or polypropylene long-leg fitted orthoses (KAFOs) with an ischial supporting lip at the time of increasing difficulty in walking. This approach to management was first introduced by Vignos and Siegel in the 1960s (Siegel *et al.* 1968; Spencer and Vignos 1962; Vignos *et al.* 1963). Their rehabilitation programme of Achilles tenotomy and provision of KAFOs resulted in an average prolongation of walking of 2 years in boys with DMD. If an equinovarus deformity is already present, and this is almost the rule after the loss of ambulation, an Achilles tenotomy may be necessary in order to fit the orthoses. This should be performed percutaneously. Compared to the surgical elongation of the Achilles tendon, this technique has the advantage of being rapid and not very painful. In our experience, almost all boys requiring ischial weight-bearing orthoses will have

sufficient Achilles tendon contractures requiring surgical intervention to fit the orthoses. In some children, serial below-knee plasters achieve sufficient correction to allow fitting of the orthoses. Some children who do not require tenotomy at first and walk regularly in their orthoses may subsequently have problems with Achilles tendon tightening and require a tenotomy later.

A small proportion of children requiring ischial weight-bearing orthoses (KAFOs) will also have significant hip flexion or ileotibial band contractures. Additional tenotomy of the hip flexors or tensor fascia lata might be required in these children. This can be combined with Achilles tenotomy in the one operation. However, in our experience, multiple level surgery makes rehabilitation more difficult because of the increased pain and decreased stability. We consider it to be necessary only when major contractures that prevent walking in KAFOs are present. In our clinical practice, only 5% of children have required ileotibial band release, and <1% have required the combination of Achilles tendon, hip flexors, and ileotibial band releases.

The benefits of rehabilitation procedures are that they result in the prolongation of walking in orthoses by around 18 months, psychological benefits, and delay in the development of progressive scoliosis (Rodillo *et al.* 1988). In our experience, KAFOs are constructed of individually moulded polypropylene and aluminium alloy and have the advantage of being strong, lightweight, and unobtrusive, as they are worn under the trousers (see Fig. 13.5). The orthoses are made the week before surgery and are available when the child is admitted for surgery. The child undergoes surgical intervention at the beginning of the week, with surgery lasting less than half an hour. The child is fitted in theatre with his night splints. The day after surgery, the child has two sessions of standing in his KAFOs, and this is progressively increased during the rest of the week. Usually, within 7–10 days, children are able to walk independently.

The mean for prolongation of independent walking from various published studies is ~24 months. Often, children will continue to be able to stand in KAFOs, often with the help of a standing frame, for a further 18–24 months after the loss of the ability to walk with KAFOs.

Because of the unpredictable course of the disease, especially shortly before the loss of independent ambulation, and the narrow window of opportunity in which to successfully rehabilitate children in KAFOs, it is vital to review boys with DMD regularly at a centre where facilities for rehabilitation are available. The advantages include the psychological preparation of the family for the loss of walking, informing them about the option of rehabilitation in KAFOs, and choosing the appropriate timing of intervention.

In our experience, the application of orthoses does not accelerate the deterioration in muscle power, and, in fact, there may be a slight increase for a time.



Fig. 13.5 A standing child in KAFOs.

Late surgery

Late surgery to correct severe equinovarus deformities in the non-ambulant phase of the disease might be required if pressure sores develop. Occasionally, surgery may be requested for cosmetic reasons in children who wish to maintain their feet in a satisfactory position and wear ordinary shoes. However, parents should be made aware of the potential risk of general anaesthesia, which, when administered for a 'cosmetic procedure' may not outweigh the benefits.

Standing frames and walkers

These are devices that allow a boy who can no longer stand unaided to achieve and maintain an upright position (see Fig. 13.6). The advantage of this equipment is that it allows the maintenance of an upright position and provides stretching of the leg joints, in addition to the psychological benefits of standing. However, these aids, in general, have limited manoeuvrability and may be difficult to introduce into the daily routine, unless a dedicated physiotherapist is available for help. As mentioned earlier, they can be used in combination with KAFOs in the later stage of standing. Modified wheelchairs have also been designed for the same purpose and have the advantage of being directly controlled by the child. However, they are much more expensive than ordinary wheelchairs. Standing may benefit the child by stretching the lower limbs, promoting a straight spine, and thereby delaying the onset of scoliosis and loading the bones which may help to delay immobility-related osteoporosis.



Fig. 13.6 A simple standing frame.

Reproduced courtesy of Dr G. M. Cochrane and the Mary Marlborough Lodge.

An extension of the standing frame is the swivel walker whereby the patient, whilst being maintained in an upright position, can progress forward by swinging forward the hips. The main problem in DMD is hip and truncal weakness so that their use is limited, compared to other orthoses such as KAFOs. Considering their cost and the limited functional benefit in children with DMD, they are very rarely used.

Surgery for scoliosis

Once the child is wheelchair-dependent, joint contractures and scoliosis soon develop. Although contractures are not a serious problem, their development does limit whatever limb movement remains and can also make dressing and lying in bed difficult. Scoliosis, on the other hand, is a serious complication. Sitting becomes difficult and uncomfortable, but, more importantly, progressive thoracic deformity restricts adequate pulmonary ventilation and aggravates respiratory problems resulting from weakness of the intercostal muscles. Respiratory impairment becomes a major threat to life and increases, once the child is wheelchair-dependent (see Fig. 13.7). It should be noted, however, that not all boys develop scoliosis; a small proportion develops hyperlordosis, and some retain more or less a normal spinal curvature. Only exceptionally is spinal curvature evident before the loss of ambulation, and, if it does occur, it is usually postural. Parents can be reassured that intervention is never required whilst the child is ambulant.



Fig. 13.7 Untreated scoliosis.

There are several ways in which the development of scoliosis can be limited or delayed. The most important one is the prolongation of ambulation and/or standing, particularly with corticosteroid treatment, followed by the adoption of a correct sitting posture. The use of spinal jackets to delay progression remains controversial, and their use should be preserved only for boys in whom spinal surgery is contraindicated. From early on, even before ambulation is lost, it is important to emphasize a habit of adopting a correct sitting position. Once confined to a wheelchair, this becomes especially important. A firm back support and special seating (for example, the Toronto seat), including a thoracic support, if needed, will help, but such measures are likely to have only a limited effect. Spinal surgery should be offered to all boys where the Cobb angle exceeds 30° and has been shown to be progressive. Each child requires a careful multidisciplinary assessment to determine their suitability for surgery. Some children with poor respiratory function may need to be established on NIV before surgery. Evidence of clinically significant cardiomyopathy should be considered an absolute contraindication for spinal surgery. In these children, referral for a



Fig. 13.8 Moulded and fitted back support.

polypropylene spinal orthosis to aid sitting is appropriate. Once the decision for spinal surgery has been made, it should be arranged as soon as possible, before the ‘window of opportunity’ for safe anaesthesia is lost (see Fig. 13.8, Fig. 13.9, and Fig. 13.10).

Spinal surgery is a major procedure that, in addition to the general complication of surgery, is almost invariably associated with substantial blood loss. This phenomenon is more severe in DMD than in other neuromuscular diseases, and it has been speculated that it might be related to smooth muscle involvement in the disease (see Chapter 10). In addition, the surgery results in an immediate mild loss of respiratory capacity, although this is counterbalanced by the loss of progressive reduction of respiratory function secondary to the scoliosis. There is no doubt that spinal surgery improves the child’s sitting posture and increases comfort. When questioned, almost all boys are pleased with the result, although self-feeding becomes impossible for some. One study, some years ago, suggested that spinal stabilization in DMD might favourably affect long-term survival. However, there is lack of evidence from

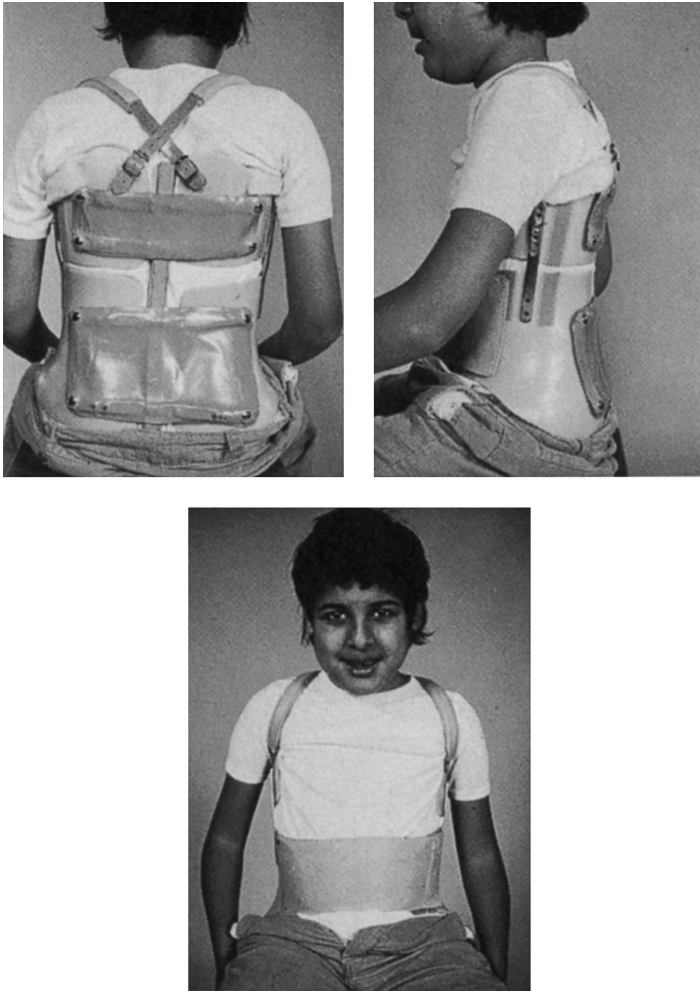


Fig. 13.9 Fitted spinal brace.

Reproduced courtesy of Dr G. M. Cochrane and the Mary Marlborough Lodge.

randomized controlled trials to support this hypothesis, and evidence from non-randomized studies is conflicting (Cheuk *et al.* 2007). A more recent observational study showed that boys who had spinal surgery and were then treated with NIV survived longer than those who did not have spinal surgery (Eagle *et al.* 2007).

As mentioned earlier, there is increasing evidence that corticosteroid use not only prolongs the ability to walk, but also reduces the risk of developing scoliosis, and therefore surgery.

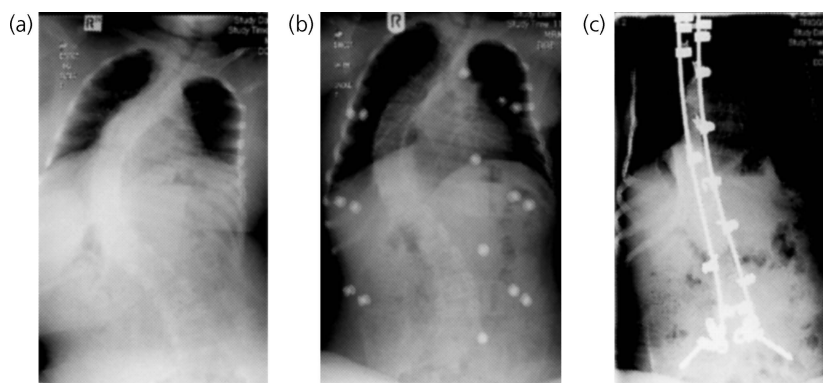


Fig. 13.10 (a) Patient with severe scoliosis. (b) Patient with scoliosis only partially corrected with a thoracolumbar brace. (c) Same patient showing good correction, following scoliosis surgery using Universal Scoliosis System instrumentation.

Reproduced courtesy of Mr Ian Lehoski.

Surgical–anaesthetic risks

Many children with DMD and BMD tolerate surgery and general anaesthesia well, but there are recognized dangers. The most serious complication is acute rhabdomyolysis which can cause renal failure, hyperkalaemia, cardiac arrhythmias, and sometimes cardiac arrest. The reaction is similar to malignant hyperthermia but does not respond to dantrolene administration, and it can lead to an exacerbation of muscle weakness. Interestingly, the risk for rhabdomyolysis appears to be higher in younger children with DMD, perhaps as a result of the better preserved muscle bulk early in life. This complication has also been reported in manifesting carriers. Potent inhalational anaesthetics, such as halothane, and depolarizing muscle relaxants, such as succinyl choline, are the usual precipitants and should be avoided. Propofol is safe and is the preferred method for general anaesthesia in DMD and BMD. Parents should be warned of this potentially serious reaction and advised to inform any non-neuromuscular specialists involved in their child's care if surgery is required.

In older boys and adults, post-operative complications, including gastric dilatation, with perforation and rarely acute gastric necrosis, have been reported. Respiratory problems, especially aspiration and chest infection, are also likely to occur post-operatively, especially when the respiratory function is already reduced.

Respiratory problems

Impaired pulmonary function is the major factor in morbidity and mortality in DMD. Over 90% of deaths result from pulmonary infection and respiratory

failure. Preservation of respiratory function and immediate treatment of respiratory infections are therefore essential elements of patient management.

At the simplest level, parents and relatives and, of course, most importantly, the boys, when they become teenagers, should be dissuaded from smoking. Vaccination against *Pneumococcus* and influenza virus at the beginning of the winter months is recommended in wheelchair-dependent children and those on corticosteroids. Well-designed respiratory exercises are valuable in helping to maintain good pulmonary function. However, these measures alone will not prevent the progressive decrease in pulmonary function. Prophylactic antibiotics are not usually recommended for the prevention of respiratory infections. However, adult patients with poor respiratory reserve should be prescribed antibiotics to keep at home, for immediate use at the first sign of an infection.

Regular pulmonary function tests, the simplest being the assessment of the vital capacity using a spirometer, are helpful in monitoring the development of significant impairment, even before problems arise, and can be a good prognostic indicator. Significant deterioration begins at around the time the boy becomes confined to a wheelchair, and measures of pulmonary function (vital capacity, and maximum inspiratory and expiratory pressures), instead of increasing with age as they do normally, remain more or less the same for some time and then in the later stages significantly decrease. Differences between predicted and observed values therefore become more marked as the disease progresses. Symptomatic respiratory failure with changes in blood gases occurs from the early to mid-teenage years and peaks in late teenage years. Today, because of proactive monitoring and early intervention with NIV when significant nocturnal hypoxaemia occurs, symptomatic respiratory failure is rare.

In non-ambulant boys, any chest infection must be treated vigorously with antibiotics and physiotherapy. If there is any suggestion that respiratory function is already impaired, then hospitalization is indicated. In older patients, the use of a suction machine to assist coughing, called an insufflator-exsufflator, can have a major impact in reducing the frequency of chest infections in patients with poor cough ability.

Assisted ventilation

Impaired respiratory function is the main cause of death in DMD, accounting for ~90% of all deaths. Respiratory failure may appear to be sudden, often precipitated by a respiratory infection in a boy whose pulmonary function is already impaired. This poses two questions. First, how can such impairment be detected before serious problems arise? Second, what can be done to alleviate this impairment?

Once a boy has become wheelchair-dependent, regular and careful review of the respiratory function at every visit is very important. Since respiratory insufficiency is first reflected in nocturnal hypoxia and hypercapnia, suggestive symptoms of this should be specifically sought for, since they may be overlooked. These include restless sleep, nightmares, morning confusion, headache, and sleepiness during the day. Often, appetite is decreased, and affected individuals fail to thrive. Later, clear signs of impaired pulmonary function become evident, including breathlessness and difficulty with speaking. Sleep hypoventilation can be confirmed by overnight oximetry. In clinical practice, we recommend regular (yearly) overnight oxygen saturation studies when the FVC falls below 40% of normal. However, the nocturnal oxygen saturation is almost invariably normal, until the FVC falls below 30%. When this occurs, oxygen saturation studies should be carried out every 6 months, and the parents, the children themselves, and other colleagues involved in the care of these patients should be told of the symptoms of nocturnal hypoventilation and its effects.

The most effective way of alleviating impaired respiratory function is nocturnal non-invasive assisted ventilation using some form of portable ventilator (NIV). This involves intermittent positive pressure with a nasal tube, mouth adaptor, or facial mask, which is well tolerated by the great majority of patients. It is important to choose the right moment for starting NIV. In particular, it is important to start the treatment not only when respiratory function is impaired, but also when abnormal morning blood gases are documented in these children. A study performed in France on the use of 'prophylactic' nocturnal ventilation in DMD children not in respiratory failure failed to show any benefit and showed it was actually detrimental. It is therefore important not only to carefully document the clinical symptoms of respiratory failure, but also to document abnormal nocturnal oxygen saturation. If early morning $p\text{CO}_2$ is abnormal, and if daytime $p\text{CO}_2$ is also abnormal, then there is strong indication for starting nocturnal ventilation as soon as possible. It has been shown that mean survival, once daytime hypercapnia develops, is only 9.7 months without respiratory assistance. A recent randomized study has demonstrated the benefit of starting nocturnal ventilation as soon as nocturnal disordered breathing is detected, compared to boys in whom the introduction of respiratory assistance is delayed.

One of the initial concerns when starting children on nocturnal NIV was related to the assumption that this treatment, initially only offered at night, would inevitably be less effective due to progressive deterioration in respiratory function. This could result in the need for longer periods of ventilation and eventually, after a few years, to 24-hour ventilation. In this instance, the

use of a tracheostomy was considered a valid alternative, but this raised problems related to maintenance and nursing care. More recently, however, various studies have shown that the use of nocturnal non-invasive ventilatory support in DMD is followed by a long period of stabilization. A study on the effects of nasal intermittent positive pressure ventilation (NIPPV) on survival in symptomatic DMD patients with established respiratory failure provides a clear example of this. Nocturnal NIPPV was applied in 23 consecutive DMD patients, with ages ranging from 14 to 26 years, who presented with diurnal hypercapnia. One- and 5-year survival rates were 85 and 73%, respectively. Interestingly, improvements of arterial blood gas tensions were maintained over 5 years, with only one case requiring ventilation during the day. Various measures of quality of life were studied and found to be equivalent to those of other groups with non-progressive disorders using the same ventilatory support (Simonds *et al.* 1998). More recently, mouthpiece ventilation has been introduced as an additional respiratory tool for those cases also requiring NIPPV during the day. The combination of nocturnal mask ventilation and the use during the day, if required, of mask or mouthpiece ventilation has resulted in very few, if any, patients requiring a tracheostomy. The mean age of survival of ventilated DMD patients has been evaluated by Bushby's group from Newcastle Neuromuscular Centre. They reported that, whilst the mean age of death in the 1980s before ventilation was introduced was around 19 years of age, this is currently 24.3 years of age, with no patients requiring a tracheostomy (Eagle *et al.* 2002). These figures do not take into account the small proportion of patients who develop an early and severe cardiomyopathy, who usually die in cardiac failure before ventilation is started. Recent collaborative data related to the mean age of survival of patients, followed in a collaboration between Great Ormond Street Hospital and Brompton Hospital, suggest a mean age of survival of 29 years, with some surviving well beyond this age (courtesy of Professor Anita Simonds and Dr Michelle Chatwin).

Cardiac management

Conduction system disease occurs very rarely in DMD patients and is most often associated with severe dilated cardiomyopathy, and only a very small proportion die suddenly as a consequence of this complication, although the change in the natural history of the condition, followed by the longer survival of DMD patients, might well have an impact on these figures.

Despite the almost universal cardiac involvement in DMD by the late teens (as demonstrated by electrocardiographic and echocardiographic studies that

show left ventricular hypokinesia and enlargement), only rarely do patients develop cardiac symptoms, probably because of the relatively modest load on the heart as a result of immobility. Hypoventilation and hypoxia can also worsen an already compromised left ventricular function.

Approximately 10–15% of DMD patients die as a result of left ventricular failure; these are usually premature deaths, most often occurring in early to mid-teenage years, but rarely much earlier. In children where the left ventricular function is declining, treatment with ACE inhibitors is recommended. With further deterioration, low doses of β -blockers are added, and, when symptomatic heart failure occurs, diuretics, such as furosemide and spironolactone, are required. Cardiac monitoring from an early age is very important; there is growing evidence for the early use of ACE inhibitors and β -blockers (Duboc *et al.* 2007). Monitoring should include a cardiac echocardiography every 2 years from diagnosis in ambulant children. After the age of 10 years, or the loss of ambulation, cardiac screening should take place yearly.

Long-term prophylactic treatment with cardiac glycosides (digoxin), although once advocated, has since been shown to have no therapeutic value and is currently not recommended. Verapamil is often used to control a variety of arrhythmias in otherwise normal individuals but is contraindicated in DMD, because it may precipitate respiratory failure and heart block. A list of the drugs commonly used in DMD is indicated in Table 13.2. Natural history data from Japan have shown improved survival with the early use of a combination of an ACE inhibitor and beta-blocker (Matsumura *et al.* 2012).

Very recently, acute fat embolism has been reported as a cause of sudden death in boys with DMD. All of the children concerned sustained minor injuries, following a fall from their wheelchairs, but, within hours, developed acute respiratory distress syndrome (McAdam *et al.* 2012b).

Table 13.2 Drugs commonly used in DMD cardiomyopathy

Problem	Drug suggested	Comment
Mild left ventricular hypokinesia and dilatation	ACE inhibitor	Small doses of β -blocker, such as carvedilol, may be added
If evidence of cardiac failure	Add diuretics	Usually furosemide and/or spironolactone
If prominent arrhythmias	Implantable defibrillator device	Consider defibrillator
If atrial fibrillation	Digoxin and anticoagulant	

ACE, angiotensin-converting enzyme.

Psychological problems

Most boys give every impression of being well adapted and of having come to terms with their disability. In fact, one expert has said:

... it is worthwhile to emphasise that the Duchenne muscular dystrophy patient, after some initial frustration, is really not suffering, has above all no pain, and is on the contrary often quite content or at least acceptingly resigned after he becomes wheelchair-bound. (Zellweger 1975)

It would, however, be entirely wrong to assume that affected boys are emotionally and psychologically unscathed by their disease. Various authors have concluded that emotional problems do occur and that, not unexpectedly, affected boys tend to be more introverted than normal children. In-depth assessment, however, may require some very careful and direct questioning in order to elicit feelings on matters such as isolation, dependency, lack of privacy, and sexual needs.

The emotional reaction of a boy to his disease varies from individual to individual, and from family to family. Paramount may be a feeling of isolation because of physical disability; this is more common in children with preserved cognitive function. Recognition that his peers are physically superior may well lead to withdrawal and depression. As the disease advances, his dependency on others and lack of personal privacy will cause more stress. A proportion may feel physically unattractive. Although some apparently deny that their inability to find sexual satisfaction is a cause of distress, others no doubt do feel this, particularly because their physical disability may preclude any relief they might obtain from masturbation. Later, they have to face the imminence of premature death. Whilst some authors state that many DMD patients accept the concept of premature death without disquiet, others have reported that a major depressive disorder or serious behavioural problems can occur as a reaction to their illness. The more emotionally disturbed tend to come from families with marked conflicts, and the behaviour of the parents and normal sibs will influence that of the affected boy. The problems are made worse if the child is ill-informed about the disease. He is more likely to be emotionally stable and better adapted to his problems if the home environment is stable, there is marital harmony, the parents are perceived as being close to each other, and there are open and frequent discussions about his problems with a frank expression of feelings. All too often, there is little communication within families about the disorder.

The parents also have to face a great many problems, and again frank discussions between themselves, as well as with health professionals, can only be beneficial. Quite apart from the emotional problems associated with coping,

there are others of a more social nature that will also produce psychological reactions. Physical handicap, especially when associated with mental handicap, may be viewed as a social stigma and source of embarrassment. As the physical incapacity increases, there will be restriction on the family's freedom and activities. The parents might find little time or opportunity to be together or to have a holiday. The husband may feel neglected or rejected because of the mother's necessary involvement with their affected son. Coupled with the fear of having another affected child, serious psychosexual problems may arise. In one survey of families, over half had serious marital problems, and a quarter had become divorced. On the other hand, in some families, the affected child has the effect of actually bringing the parents closer together.

Finally, unaffected sibs are not excluded from the emotional problems that may arise within the family. Overprotection and pampering of an affected boy may result in jealousy and resentment among sibs. Older sisters may adopt a maternal or protective role, yet, at the same time, harbour increasing concern about their possibly having an affected son.

Emery and Pullen (1984) recognize seven stages in the evolution of a genetic disease within a family, each being associated with different emotional and psychological responses in different members of the family:

- 1 positive family history;
- 2 abnormality noticed by parents;
- 3 abnormality confirmed by family practitioner;
- 4 diagnosis first made/coping process begins;
- 5 resolution/adaptation;
- 6 chronic handicap/progression;
- 7 death/grieving.

At stage 1 (when there is a family history of DMD), those who see themselves as being at risk of having an affected son are likely to be anxious and concerned. With new improved methods of carrier detection, reassurance is often now possible. Otherwise, the medical and genetic aspects of the problem will need to be discussed in detail, perhaps on several occasions, until an acceptable course of action is reached. All too often, there is a considerable delay between the time when the parents first notice that something appears to be wrong with their son and the time when this is agreed by the family practitioner and the diagnosis is established. Most couples interviewed find this period of uncertainty one of the most trying and unsettling. Unfortunately, current evidence suggests that there is no increased awareness by family practitioners and paediatricians of the possibility of muscular dystrophy. The age at diagnosis of DMD

has not changed significantly in the last decade. Stage 4 involves the emotional reaction to the diagnosis and the beginning of the coping process. Stages 5 and 6 involve the reactions of the patient and his parents and sibs to the disease. Parents must be encouraged to take time off and have regular times set aside for themselves. Organizations, such as the Muscular Dystrophy Campaign in Britain, through local set-ups, can provide support and advice and help to reduce feelings of isolation. Open and frank discussions between all members of the family should be encouraged, including the affected boy himself. As Pullen, a psychiatrist experienced in this field, has stated:

. . . The physically handicapped child must be allowed to talk about his frustrations, disappointments, depression and anxieties for the future. Many people, including parents, do not allow the child to talk about these areas for fear of putting ideas into his head. The ideas certainly are there already but most children are denied the opportunity of communicating them to others. This may make them feel more isolated and abnormal because it prevents others from empathizing accurately with their position. (Emery and Pullen 1984, p. 122)

About a third of parents we have interviewed had great difficulty in talking to each other about the disease.

Finally, at stage 7, parents again should be encouraged to talk honestly about their emotional reactions: their distress, despair, and perhaps anger. The problems of bereavement in muscular dystrophy have been analysed in detail by several authors. Ideally, counselling should not end with the death of the patient but should be available to close relatives, until grieving has passed. It is doubtful if the sense of loss and the attendant grief will ever completely pass away. With time, however, there is often a sense of relief after all the years of anxiety and concern. This is natural, and not a reason for feeling guilty.

Following these considerations, the question arises as to the best way to provide appropriate psychological and emotional support to the DMD patient and, in particular, if and when psychological support should be offered to the family. Of key importance in this is the attitude and empathy of the staff (medical, nursing, family care officers, and physiotherapy) involved in the care of these patients. A multidisciplinary approach is also very important, as often one colleague might sense or realize a particular problem not necessarily evident to others.

What we suggest in clinical practice is an early and precise diagnosis. The communication of the diagnosis should be performed by a senior physician with experience not only in DMD, but also in techniques of communication. If at all possible, a specialist nurse should also be present and offer support to the family, following the diagnosis. This is helpful not only to introduce the family to various non-medical issues (ranging from support at school to disability

allowance), but also because it usually takes a long time, often many years, for families to fully understand the implications of a diagnosis like DMD. These professionals can therefore provide important additional information to families, with feedback to the physicians, by performing home visits after the diagnosis has been made and advising the family on practical matters such as home modifications.

In order for families to be fully informed of the different problems encountered at different stages of the disease, but also of the various solutions and help that are available, we found it very helpful to run clinics entirely dedicated to DMD patients and focused on specific problems that characterize a particular stage of the disorder. In the ambulant phase, children are followed in a dedicated clinic in which they are recruited for the rehabilitation programme using KAFOs. Following their loss of independent ambulation, patients are channelled to a combined neuromuscular/orthopaedic clinic where the issue of scoliosis is discussed and individuals are given the opportunity to interact with other patients who have already undergone surgical correction of the spine. In the later stages of the disease, patients with respiratory problems are channelled to a dedicated combined neuromuscular/respiratory clinic for ventilated individuals. Bereavement counselling is offered to all families, following the death of the affected individual. With this pragmatic and dedicated approach, in which the key physician and his or her own team also act as counsellors to the family, informing them of both the problems and solutions available, serious psychological problems are rare. Depression may, however, still develop, especially in adolescents. These cases should be referred to an experienced clinical psychologist or, if required, to a psychiatrist aware of the problems of DMD.

Educational and social needs

The first question to be answered is whether an affected boy may require special schooling. In a few cases associated with severe mental handicap, this may be indicated when the parents find management difficult. In most cases, however, boys can derive considerable benefit from attending a normal school where the teachers can be very helpful, once the problem has been explained. The several problems that may affect a boy's educational ability include progressive motor difficulties that make the acquisition of new skills more difficult and frequent absence from school with declining physical condition. Affected boys also tire easily and may lack initiative and motivation.

Attention should focus on a boy's positive abilities. A proportion will be academically orientated. But their physical incapacity, by the time they reach

senior school, can present a serious problem, and further education and employment prospects are limited but are now being increasingly recognized and addressed. Several of our patients, since the availability of NIV, have completed university degrees. Others work part-time as information technology officers and engineers. Some patients are artistically inclined. Over the years, one of us has made a collection of drawings and paintings by patients, and their skill and ability are frequently a source of admiration.

As the disease progresses and boys become confined to a wheelchair, day schools that cater specially for the physically handicapped can be an attractive proposition. In such an environment, they will feel less isolated and more able to share feelings and emotions with fellow sufferers. Most parents prefer to have their child in a day school with other handicapped children, rather than in an institution away from home.

At home, consideration should be given to the time when the DMD patient will be unable to walk unaided, and appropriate plans made. Ramps to take a wheelchair may be required. A ground-floor bedroom/study is ideal, with a TV, hi-fi, computer, and other devices of his choosing. In this way, he will have a place that he can consider his private sanctum in which to entertain friends and be on his own if he chooses.

There is a vast array of aids, including wheelchairs, available for the physically handicapped. In several countries, including Britain, some of these can be obtained through the National Health Service, at no cost to the family. It would not be appropriate to deal with these matters here. However, there are several publications that are of considerable practical value and provide information not only on aids, but also addresses of where to seek help and advice—these can be obtained through the national support groups.

As the natural history of the condition changes and improves, it is extremely important that affected boys with normal intelligence are encouraged to succeed at school. Our experience of caring for adults with DMD at Queen Square has shown that those boys who have grown up with parents who have a positive attitude are most likely to achieve their full potential. Some boys do well at university, learn to drive, and live independently with carers. A very small minority settle down with partners and have children of their own. For many affected adults, the quality of life is perceived as good. Advances in the Internet and electronic technology have had a major impact in reducing isolation for DMD boys and adults. Advanced care planning and identifying a clear point of access for emergencies is an important aspect of adult DMD care. Interestingly, in our experience, almost all affected adults express a wish for full medical care and resuscitation, in the event of developing a critical illness.

Therapeutic research studies

The assessment of the possible therapeutic benefits of a drug in DMD presents a number of problems that have to be considered. Few drug trials in the past have been properly designed, although this is now a very rapidly evolving field due to the improved understanding of the pathophysiology of the condition.

Early drug trials

During the last century, there have been many drug trials in DMD. Some of the drugs that have been tried and the basis for their use are summarized in Table 13.3. Many of the early studies were ill-designed. Often, an initial study claiming benefits for a particular drug was subsequently refuted by a better designed and better controlled study.

Evaluation of drug trials

A detailed critical review of the many past therapeutic trials in DMD was carried out in 1980, and a system devised of awarding a 'quality score' for each report, with a point for each of the following criteria: careful selection and definition of cases, adequate controls, objective ('blind') study, assessment other than by simple clinical ratings, and for a trial lasting longer than 2 years. Of 34 trials published, not one was awarded five points, and, in half, there was no score at all, which means that even the definition of the cases studied was not clear (see Table 13.4). Trials with a score of less than three are seriously flawed and have the danger of raising false hopes in both the patient and his family. The choice of outcome measures was also often problematic due to a lack of properly conducted studies in which the assessment and variability of these measures was considered.

Design of drug trials

There are a number of general points to be considered in designing a DMD drug trial. Patients must be carefully selected on the basis of accepted clinical, biochemical, and genetic diagnostic criteria for the disease. The inclusion of patients with BMD, for example, might give the mistaken impression of slowing the course of the disease. The patients should also be old enough and capable of ambulating and cooperating with the assessment. Ideally, they should therefore be aged between 5 and 10 years, although the upper age limit has now been shifted forward due to the use of corticosteroids. The trial should be 'blind', unless the nature of the treatment (for example, surgery) or the occurrence of unusual side effects makes this impossible. Ideally, it should be double-blind, with neither the patient and his family nor the investigator knowing who

Table 13.3 Drugs (arranged alphabetically) used in various therapeutic trials in DMD

Drug	Basis for use	Trial
Allopurinol	Increases nucleotide formation believed to be depleted in dystrophic muscle	1976
Amino acids	Deficiency of muscle proteins	1953
Aminoglycoside	Read-through of stop codons	2001
Anabolic steroids	Anabolic effect	1955
Aspirin, propranolol, etc.	Counteract proposed defect in biogenic amine metabolism	1977
Azathioprine	Immunosuppression	1993
Calcium blockers	Reduce muscle intracellular calcium	1982
Catecholamines	Counteract proposed defect in muscle sympathetic innervation	1930
Ciclosporin	Immunosuppression	1993
Coenzyme Q	Possible benefit in murine dystrophy	1974
Creatine	Deficiency of muscle creatine	2000
Dantrolene	Inhibits release of calcium from sarcoplasmic reticulum	1983
Digitalis and other cardiac glycosides	Prevent progressive cardiomyopathy	1963
Glycine	Stimulates muscle creatine synthesis	1932
Growth hormone	Anabolic effect	1973
Growth hormone inhibitor	Growth hormone deficiency ameliorates disease	1984
Ketoacids	Reduce muscle protein degradation	1982
Leucine	Increases protein synthesis	1984
Nucleotides (for example, laevadosin)	Replacement of nucleotides believed to be depleted in dystrophic muscle	1960
Oestrogens	Anabolic effect	1972
Oxandrolone	Anabolic effect	1997
Pancreatic extract	Possible benefit in murine dystrophy	1976
Penicillamine	Possible benefit in avian dystrophy	1977
Prednisolone	Anabolic and immunosuppressive effect	1974
Protease inhibitors	Possible benefit in murine dystrophy	1984
Superoxide dismutase	Removal of superoxide radicals associated with membrane damage	1980
Testosterone	Anabolic effect	1955
Thyroxine	Thyroxine depresses SCK	1964
Vasodilators	Counteract proposed defect in muscle microcirculation	1963
Vitamin B ₆	Vitamin B ₆ -deficient rats develop myopathy	1940
Vitamin E	Vitamin E-deficient animals develop myopathy	1940
Zinc	Membrane 'stabilizer'	1986

Table 13.4 Scoring of 34 drug trials in DMD (1940–79)

Score	Number of trials
0	17
1	7
2	3
3	3
4	4
5	0

Source: data from Dubowitz, V. and Heckmatt, J., Management of muscular dystrophy: Pharmacological and Physical Aspects, *British Medical Bulletin*, Volume 36, Issue 2, pp. 139–144, Copyright © 1980 Oxford University Press.

is taking the drug and who is taking a placebo. It may be difficult to convince parents of the value of this method, because, if they already believe the drug could be effective, it would mean, in some cases, denying the possible benefits of treatment for the duration of the trial. For this reason, a cross-over, double-blind study has an advantage and also requires fewer patients. The placebo must have the same appearance, texture, and taste as the drug. Furthermore, the effects of the drug may take some time to wear off (the so-called ‘washout’ effect) and so vitiate the results of a cross-over study. If a placebo study is not feasible, a possibility is to use ‘natural history controls’ for comparisons, that is, data collected from known cases over a period of time. However, an important limitation of this choice is that the natural history study of DMD is rapidly evolving, also due to change in management strategies and improved standards of care.

Usually, novel medicines are first tried in healthy volunteers (phase Ia studies). If safe in normal volunteers, the next step is to assess their safety in a small group of DMD boys, often in ascending doses (the so-called phase Ib trial).

The possible beneficial effects may be assessed in regard to prolonged survival, prolonged ambulation, or the slowed or arrested progression of muscle weakness. Measurements of muscle strength are often performed, and a lot of emphasis has been placed on this aspect of the problem in the past (see Table 13.5). Because there is a subjective element in determining muscle strength and, to some extent, functional ability, these are best assessed by one examiner. There is also value in using an ergometer (myometer, dynamometer). One commonly used device is the handheld, sensitive dead-beat electronic device produced by CITEC, B. V., The Netherlands. The unit has a pressure pad and a digital readout display that indicates the maximum force applied by the examiner in resisting the actual contraction of the patient’s muscles. The operating range is 0.1–30 kg, and it has a repeatability error of <1% (see Fig. 13.11).

Table 13.5 Methods for assessing the possible beneficial effects of a drug in DMD

Muscle strength
MRC grading (0–5)
Ergometry (kg)
Respiratory muscle strength
Functional ability
Vignos grade (1–10)
Hammersmith motor ability score (0–40)
‘CIDD’ grade for upper limbs (1–6)
Biochemical
SCK
Urinary creatine/creatinine, 3-methylhistidine, dimethylarginines
Muscle pathology and dystrophin expression

**Fig. 13.11** Example of a handheld myometer and its use to evaluate knee extension in a child with DMD.

Nevertheless, it has become increasingly clear that there is not a linear correlation between strength and function in DMD boys. The early phases of the disease are characterized by marked changes in muscle strength, whilst function changes less dramatically; on the contrary, in the late ambulant phases, small changes in muscle strength are associated with significant functional advantages. It is because of this that it is preferred that functional outcome

measures (such as functional scales) or other measures of function, such as timed tests or 6-minute walk tests, are primary outcome measures for the condition, not strength.

In recent years, several investigators have measured various biochemical parameters as a means of assessing any possible improvement. Urinary excretion studies are obviously more acceptable than repeated blood sampling, though the former require to be very carefully controlled for age, sex, and diet. There is little value in repeated muscle biopsies to evaluate morphological changes, because of the marked variability of pathology due to muscle sampling. A muscle biopsy for dystrophin analysis might, however, be required in drug trials focused on the replacement or upregulation of muscle proteins (such as dystrophin or utrophin, for example). Muscle imaging using MRI has also been used to demonstrate the skeletal muscle involvement of DMD boys. A few longitudinal studies have also been published that clearly demonstrate the progressive nature of morphological changes, affecting more the proximal, rather than the distal, muscles and show the progressive and severe replacement of skeletal muscle tissue by fat. It is likely that muscle MRI will be used in the future as a surrogate measure of response to therapy, as it provides compelling evidence on the anatomical status of the muscles.

Another test that has been studied both in animal models (dystrophic mice and dystrophic dogs) and in DMD and BMD patients is MRS. In particular, phosphorus MRS has demonstrated an abnormal pH and a lower phosphocreatine/inorganic phosphorus ratio in patients and the animal models, indicating a profound metabolic disturbance of the dystrophin-deficient muscle.

Statistical considerations

A drug trial should be designed in such a way as to avoid errors of suggesting a beneficial effect when none exists or, alternatively, concluding that there is no effect when, in fact, the drug arrests or slows the disease process. To detect a therapeutic effect that is small, such as a gradual slowing of the disease process, requires a prolonged trial involving a large number of patients. Conversely, a marked therapeutic effect would be detectable in a shorter time and would need fewer individuals. In this regard, a helpful parameter to be determined is the so-called power of the trial. If the rate of decline in untreated boys is r_1 (with an SD) and in treated boys is r_2 , then the **standard difference** (delta, Δ) is:

$$\Delta = (r_1 - r_2) / \text{SD}$$

The power of a trial is the probability of detecting a difference in the two rates that is statistically significant ($p < 0.05$) and can be calculated for different

numbers of individuals and for trials of different durations. Based on data from 114 untreated boys with DMD followed for a year, power curves have been derived by Brooke *et al.* (1983). These investigators found that the rate of decline in untreated boys was 0.4 units (of muscle strength) per year (SD, 0.39). If, after a year, a drug slowed the disease to 25% of its original rate of progression, then:

$$\Delta = (0.4 - 0.1)/0.39 = 0.77$$

To detect such a difference with a 95% probability would require a study involving at least 40 individuals in each group. On the other hand, if a drug actually arrested the progression of the disease, about 25 individuals in each group would be required (see Fig. 13.12).

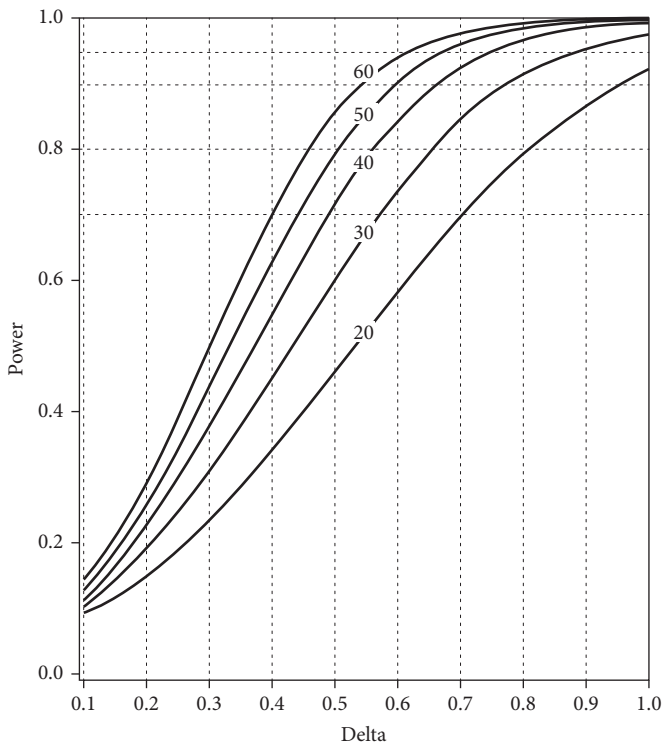


Fig. 13.12 Power curves ($p < 0.05$, one-tail) for drug trials lasting a year for various values of the standard difference (delta).

Reproduced from Brooke, M. H., *et al.*, Clinical investigation in Duchenne dystrophy: 2. Determination of 'power' of therapeutic trials based on the natural history, *Muscle and Nerve*, Volume 6, Issue 2, pp. 91–103, Copyright © 1983, with permission from John Wiley and Sons, Inc.

By using more than one set of measurements, the power of a trial study can be increased, thereby decreasing the number of individuals needed and/or the duration of the trial. However, the rate of progression is clearly not the same in all boys. It might therefore be best to consider each boy as being his own control by comparing rates of progression before and after treatment. Alternatively, groups of boys may be compared who, prior to a trial, showed similar rates of progression.

Taking into account the 'power' of a study, the criteria of a good trial in DMD should be:

- ◆ inclusion only of patients with a clearly established diagnosis of the disease;
- ◆ carefully matched control group;
- ◆ 'blind' study;
- ◆ assessment by several different methods;
- ◆ determination of an acceptable 'power' level, and therefore the number of individuals to be studied and the duration of the trial.

The assumption underlying the discussion so far is that quite a large number of patients will be involved in a study. Each individual investigator, however, is unlikely to have access to many patients, and therefore collaboration between different centres is necessary to produce data for a so-called 'multicentre trial'. Uniformity is then essential, both in the design and execution of the trial, particularly with regard to the clinical assessment of the possible effects of a drug. The alternative is to study smaller groups of patients, but then, as we have seen, only a comparatively large effect would be detected (see Table 13.6).

A useful overview of problems involved in drug trials in DMD can be found in Wolf and Lewis (1985), which gives the proceedings of a 1984 colloquium sponsored by the Muscular Dystrophy Association of America. Some other issues have been discussed at a series of the ENMC workshop on therapeutic trials in DMD (Dubowitz 2000; Mercuri *et al.* 2008).

Recent drug trials: glucocorticoids

No curative pharmacological treatment is yet available for DMD, but, of all the medications studied so far, glucocorticoids appear to be the most effective in slowing down the disease process. The precise mechanism by which glucocorticoids increase strength in DMD is not known, especially considering their negative effect on normal skeletal muscle (steroid myopathy). On the contrary, corticosteroids have an anabolic effect on DMD muscle. Among the possible effects in dystrophic muscle are the inhibition of muscle proteolysis, stimulatory effect on myoblast proliferation, anti-inflammatory/immunosuppressive

Table 13.6 Numbers (rounded off) of individuals (controls and treated) required in a randomized trial where there is a 95% probability ('power') of detecting a significant difference ($p < 0.05$; $p < 0.01$) for various values of Δ . In each case, numbers are roughly four times the required number for a cross-over trial

Δ	One-tail		Two-tail	
	$p < 0.05$	$p < 0.01$	$p < 0.05$	$p < 0.01$
0.1	2 165	3 154	2 599	3 563
0.2	541	788	650	891
0.4	135	197	162	223
0.6	60	88	72	99
0.8	34	49	41	56
1.0	22	31	26	36
1.2	15	22	18	25
1.4	11	16	13	18
1.6	8	12	10	14
1.8	7	10	8	11
2.0	5	8	6	9

effect with a decrease in muscle T-cells, and muscle fibres focally invaded by lymphocytes. In a study using the *mdx* mouse model, one of the authors (Muntoni) showed that treated mice demonstrated an overexpression of a cohort of genes relating to metabolism and proteolysis, alongside the differential expression of genes relating to calcium metabolism. Treatment did not increase muscle regeneration, reduce the number of infiltrating macrophages, or alter utrophin expression or localization. However, in the treated *mdx* soleus muscle, the percentage of slow fibres was significantly lower, compared with untreated controls, indicating a preservation of a more normal muscle fibre type.

There are now more than two decades of experience with these drugs, and the individual studies are listed in Table 13.7. The most commonly used steroid preparations are prednisone, its hydroxylated form prednisolone (equipotent in glucocorticoid effect to prednisone), and deflazacort, an oxazoline derivative of prednisolone. One mg of prednisone is equivalent in anti-inflammatory (glucocorticoid) effect to 1.2 mg of deflazacort. This latter drug has the advantage of having a lower incidence of weight gain, but other side effects are similar to those of prednisolone, and, in addition, an increased incidence of cataracts has been reported in patients treated with this drug, when compared to prednisolone.

Since all these studies have used different steroids or regimens and although the overall results seem to suggest a slowing of the disease process, at least in the

Table 13.7 Glucocorticoid trials in DMD

Authors [†]	Design	N	Age (years)	Glucocorticoid regimen	Treatment period	Outcome	Comments
Drachman 1974	Open	14	4–10.5	Prednisone 2 mg/kg/day for 3 months; then 2/3 dose on alternate days	3 weeks–28 months	Improvement	Side effects in four
Siegel 1974	Double-blind	14	6–9	Prednisone 5 mg/kg on alternate days	24 months	No benefit	
Brooke 1987	Open	33	5–15	Prednisone 1.5 mg/kg/day	6 months	Improvement	Six dropouts
DeSilva 1987	Open	16	3–10	Prednisone 2 mg/kg/day for 3 months; then 2/3 dose on alternate days	1–11 years	Walking prolonged by 2 years	Excessive weight gain in 12; cataracts in two
Mendell 1989	Randomized, double-blind	103	5–15	Prednisone 0.75 mg/kg/day; prednisone 1.5 mg/kg/day	6 months	Improved at 3 months; then stabilization	30% of boys had >20% of weight gain
Fenichel 1991	Double-blind	103	5–15	Prednisone 1.25 mg/kg/alternate day; prednisone 2.5 mg/kg/alternate day	6 months	Improved at 3 months	Similar side effects on daily and alternate day regimes
Fenichel 1991b	Open	92	5–15	Prednisone 0.75 mg/kg/day	2 years	Stabilization for 2 years; prednisone 0.65 mg/kg/day least effective dose	Cataracts in ten; glycosuria in ten; significant weight gain
Griggs 1991	Randomized	99	5–15	Prednisone 0.3 mg/kg/day; prednisone 0.75 mg/kg/day	6 months	Strength improved at 10 days	30% of boys had >20% of weight gain
Mesa 1991	Double-blind	28	5–11	Deflazacort 1 mg/kg/day	9 months	Improved till 6 months; then stable	35% cushingoid; no significant weight gain

Griggs 1993	Randomized	107	5–15	Prednisone 0.75 mg/kg/day; azathioprine 2.5 mg/kg/day	18 months; 12 months	Strength and function improved	No additional benefit of azathioprine
Sansome 1993	Open	32	6–14	Prednisolone 0.75 mg/kg/day, for 10 days/months (10 days on, 20 days off)	18 months	Strength improved at 6 months; slow decline at 18 months	Fewer side effects, but 26% of boys had >20% of weight gain
Angelini 1994	Randomized	28	6.5–9	Deflazacort 2 mg/kg/alternate days	24 months	Stabilization of strength	70% had >20% of weight gain; one pathological fracture
Backman 1995	Double-blind, cross-over	37	4–19	Prednisone 0.3 mg/kg/day	12 months	Stabilization for 1 year	Weight gain in 29%
Bonifati 2000	Randomized	18	5–14	Prednisolone 0.75 mg/kg/day; deflazacort 0.9 mg/kg/day	1 year	Prednisolone and deflazacort equally effective	Weight gain more significant in prednisolone group
Biggar 2001	Open	30	7–15	Deflazacort 0.9 mg/kg/day	3.8 years (\pm SD 1.5)	Ambulation prolonged; FVC preserved	Cataracts in 30%
Reitter 1995 (data reported in Dubowitz 2000)	Double-blind	100	5, till ambulant	Prednisolone 0.75 mg/kg/day; deflazacort 0.9 mg/kg/day	2 years	Muscle function stabilized	Excessive weight gain in prednisolone group; cataracts in 27% of deflazacort group

[†] Full references can be found in Manzur (2001).

Reproduced from Manzur, A. Y., Medical management and treatment of Duchenne muscular dystrophy, Emery A. E. H. (Ed), *The muscular dystrophies*, Oxford University Press, Oxford, Copyright © 2001. By permission of Oxford University Press.

short term, there is a need for a large coordinated study, using the same steroid and with the same regime, to determine if there might also be any possible long-term benefit. One of the authors has used glucocorticoids at the time of functional deterioration of children with DMD, using a treatment regime that minimizes the long-term side effects (0.75 mg of prednisolone, 10 days on and 10 days off).

Growth hormone inhibitors

Growth hormone inhibitors were proposed, following a report in the 1980s of a family with DMD, in which the eldest affected boy was much less severely affected than his brothers but who also suffered from growth hormone deficiency (see Fig. 13.13). One of us has also seen a sporadic case with DMD and growth hormone deficiency, with no dystrophin expression in the muscle, who walked until the age of 18 years. This led to the suggestion that treatment with growth hormone inhibitors might be effective in DMD. Unfortunately, this has not proved to be so. However, these observations do raise a number of intriguing questions regarding the role of growth hormone in the evolution of the disease process.

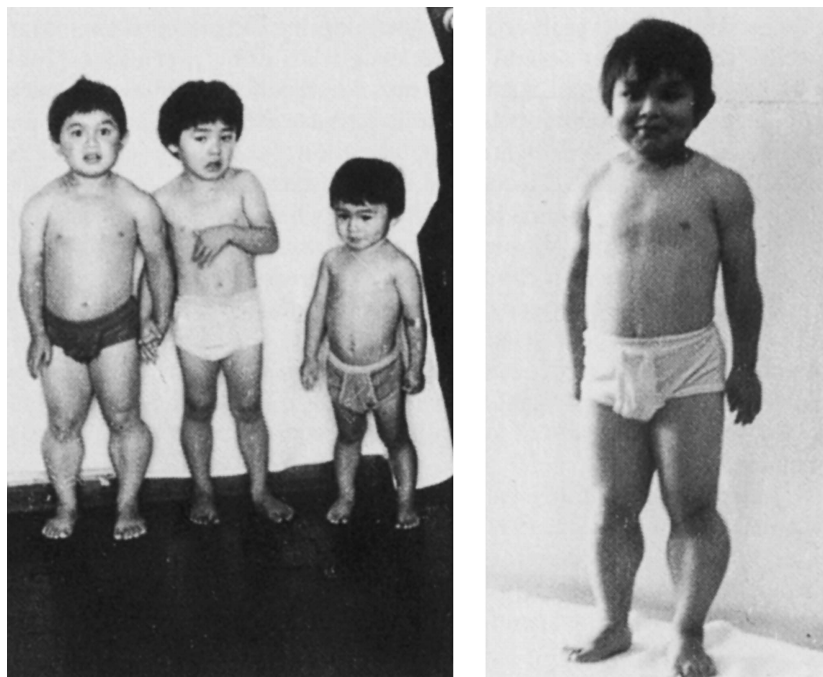


Fig. 13.13 (Left) A boy with DMD and growth hormone deficiency, aged 13, and his two younger affected brothers, aged 5 and 3. (Right) Proband, aged 18.

Image courtesy of Dr Mayana Zatz.

Oxandrolone

A pilot study in 1997 of oxandrolone (an anabolic steroid) in boys with DMD suggested some possible improvement in muscle strength, compared with natural history controls. But further double-blind studies have failed to confirm this, and oxandrolone cannot be recommended.

Azathioprine and ciclosporin

The mechanism of action of glucocorticoids is unknown, but, in view of their immunosuppressive role, other immunosuppressive drugs have been tried, including azathioprine. It was tested in a randomized controlled trial of prednisolone and azathioprine but failed to have a beneficial effect.

A beneficial effect of ciclosporin on strength (tetanic force and maximum voluntary contraction strength in the tibialis anterior muscle) has been documented after 2 months of ciclosporin therapy, but this positive effect was largely lost, following a further 2 months' washout phase. However, a recent large randomized, placebo-controlled, multicentre trial definitively found that ciclosporin failed to improve muscle strength or function in DMD boys and is therefore not recommended.

Creatine

Creatine is converted to phosphocreatine in the muscle where it provides energy in the form of ATP. A modest increase was reported in an early study, but a further large controlled trial failed to demonstrate any clinical benefit.

Aminoglycosides

The administration of gentamicin has been reported to restore dystrophin expression in skeletal muscles of *mdx* mice. The mechanism of action of this aminoglycoside antibiotic is to cause misreading of the RNA code, allowing the insertion of alternative amino acids at the site of stop codons. This therefore can theoretically induce a read-through of non-sense mutations. The application of this approach to DMD is limited, as only a minority of patients carry stop codons in the dystrophin gene, and trials of the antibiotic in affected boys have been disappointing. Furthermore, this antibiotic has serious oto-nephrotoxic side effects. Possibly another antibiotic with the same molecular effect, but less toxicity, might prove beneficial (for example, negamycin). Newer drugs designed to read through non-sense mutations, without the side effects of aminoglycosides, have been developed and are in evolution. A large international phase IIb randomized controlled trial of ataluren has recently been completed and will be followed by a phase III open-label study (see Table 13.8).

Table 13.8 A list of recently completed or currently active clinical trials

Drug	Design	Type of dystrophy	Primary outcome measures	Results	Other information
Coenzyme Q10 and lisinopril	OL, randomized	DMD/BMD	MPI	In progress	< http://ClinicalTrials.gov/show/NCT01126697 >
Revatio® (sildenafil)	MC, DB, randomized, PC	DMD/BMD	Cardiac left ventricular end-systolic volume by cardiac MRI	In progress	< http://ClinicalTrials.gov/show/NCT01168908 >
Tadalafil and sildenafil	Randomized, OL	DMD	Changes in muscle tissue oxygenation by NIRS and Doppler	Completed	< http://ClinicalTrials.gov/show/NCT01359670 >
Tadalafil	MC, DB, randomized, PC	BMD	Changes in muscle tissue oxygenation by NIRS	Completed	< http://ClinicalTrials.gov/show/NCT01070511 >
Carvedilol	Randomized, OL	DMD	Cardiac damage (elevation of plasma cTnI)	In progress	< http://ClinicalTrials.gov/show/NCT00606775 >
Perindopril	Randomized, OL	DMD	Survival at 10 years	In progress	< https://www.clinicaltrialsregister.eu/ > Number: 2008–003856–32
Ramipril versus carvedilol	Randomized, OL	DMD	Left ventricular ejection fraction, left ventricular volumes, and LGE by MRI, myocardial tissue by cardiac US	In progress	< http://ClinicalTrials.gov/show/NCT00819845 >
ACE inhibitor and β-blocker	MC, DB, randomized, PC	DMD		In progress	< https://www.clinicaltrialsregister.eu/ > Number: 2007–005932–10

Idebenone	MC, DB, randomized, PC	DMD	Peak systolic radial strain of the left ventricle by colour Doppler myocardial imaging	Completed	< http://ClinicalTrials.gov/show/NCT00654784 >
CRD007	OL, single centre	DMD, BMD	Safety, efficacy	Completed	< http://ClinicalTrials.gov/show/NCT01540604 >
Ataluren (PTC124)	DB, randomized, PC	DMD/BMD with stop codon mutations	6MWD	Completed	< http://ClinicalTrials.gov/show/NCT00592553 >
	Single group, OL, safety study	Previously treated patients with DMD/BMD	Safety	Completed	< http://ClinicalTrials.gov/show/NCT00759876 >
				In progress	< http://ClinicalTrials.gov/show/NCT01557400 >
				In progress	< http://ClinicalTrials.gov/show/NCT01247207 >
Gentamicin	Single group, OL, safety study	DMD subjects with stop codon mutations	Safety	Completed	< http://ClinicalTrials.gov/show/NCT00451074 >
GSK2402968	DB, randomized, PC	Ambulant DMD with mutations eligible for skipping exon 51	6MWD	Completed	< http://ClinicalTrials.gov/show/NCT01153932 >
	DB, randomized, PC	Ambulant DMD with mutations eligible for skipping exon 51	6MWD	Completed	< http://ClinicalTrials.gov/show/NCT01254019 >
	DB, randomized, parallel assignment	Ambulant DMD with mutations eligible for skipping exon 51	6MWD	In progress	< http://ClinicalTrials.gov/show/NCT01890798 >

Table 13.8 (continued) A list of recently completed or currently active clinical trials

Drug	Design	Type of dystrophy	Primary outcome measures	Results	Other information
GSK2402968	DB, randomized, parallel assignment, multiple doses	Non-ambulant DMD with mutations eligible for skipping exon 51	Pharmacokinetics variables	Completed	< http://ClinicalTrials.gov/show/NCT01128855 >
	OL, extension study; safety/efficacy	Ambulant DMD with mutations eligible for skipping exon 51	6MWD	In progress	< http://ClinicalTrials.gov/show/NCT01480245 >
AVI-4658 (PMO; eteplirsen)	OL, dose-ranging	Ambulant DMD with mutations eligible for skipping exon 51	Safety dystrophin restoration	Completed	< http://ClinicalTrials.gov/show/NCT00844597 >
	OL, dose-ranging	Ambulant DMD with mutations eligible for skipping exon 51	Dystrophin restoration	In progress	< http://ClinicalTrials.gov/show/NCT01540409 >
	DB, randomized, parallel assignment, dose-ranging	Ambulant DMD with mutations eligible for skipping exon 51	Dystrophin restoration	Completed	< http://ClinicalTrials.gov/show/NCT01396239 >
PRO044	OL, non-randomized	Ambulant DMD with mutations eligible for skipping exon 44	Safety/dystrophin expression	Completed	< http://ClinicalTrials.gov/show/NCT01037309 >

Mini-dystrophin	DB, randomized, parallel assignment, dose-ranging	DMD boys	Safety	Completed	< http://ClinicalTrials.gov/show/NCT0428935 >
Mesoangioblasts	OL, non-randomized	Ambulant DMD with HLA-compatible sibling (donor)	Safety	In progress	< https://www.clinicaltrialsregister.eu/ > Number: 2011-000176-33
High-dose prednisone	Efficacy, high dose, weekly versus daily	Ambulant DMD	Quantitative measurement system	Completed	< http://ClinicalTrials.gov/show/NCT00110669 >
ACE-031	DB, PC, randomized, dose-ranging, safety	Ambulant DMD	Safety	Completed	< http://ClinicalTrials.gov/show/NCT01099761 >
Creatine monohydrate and glutamine	DB, PC, randomized	Steroid-naive, ambulant DMD	Quantitative testing	Completed	< http://ClinicalTrials.gov/show/NCT00016653 >
Daily pentoxifylline	DB, PC, randomized, efficacy	Ambulant DMD	Quantitative muscle strength	Completed	< http://ClinicalTrials.gov/show/NCT00243789 >
Oxatamide	Pilot, OL	Steroid-naive, ambulant DMD	Quantitative muscle strength	Completed	< http://ClinicalTrials.gov/show/NCT00033813 >
Oral glutamine	DB, PC, randomized	Ambulant DMD	Efficacy, walking speed	Completed	< http://ClinicalTrials.gov/show/NCT00296621 >
Coenzyme Q10 and prednisone	MC, DB, PC, randomized, efficacy	Ambulant DMD		Completed	< http://ClinicalTrials.gov/show/NCT00033189 > < http://ClinicalTrials.gov/show/NCT00308113 >
Idebenon	OL, single arm, safety/ efficacy	DMD		Completed	< http://ClinicalTrials.gov/show/NCT00654784 > < http://ClinicalTrials.gov/show/NCT00758225 >
	DB, PC, randomized, safety/efficacy	DMD		Completed	< http://ClinicalTrials.gov/show/NCT01027884 >
Epigallocatechin-gallate	DB, PC, randomized	Ambulant DMD	Safety	In progress	< http://ClinicalTrials.gov/show/NCT01183767 >

Table 13.8 (continued) A list of recently completed or currently active clinical trials

Drug	Design	Type of dystrophy	Primary outcome measures	Results	Other information
IGF-1	IGF-1 and steroids versus steroid treatment	Ambulant DMD	6MWD	In progress	< http://ClinicalTrials.gov/show/NCT01207908 >
Flavocoxid	OL, single arm, safety	Ambulant DMD	Safety	Completed	< http://ClinicalTrials.gov/show/NCT01335295 >
L-arginine	OL, single arm, safety	Ambulant DMD	Safety	Completed	< http://ClinicalTrials.gov/show/NCT01388764 >
AAV1-gamma-sarcoglycan gene therapy	OL	LGMD2C	Safety	Completed	< http://ClinicalTrials.gov/show/NCT01344798 >
Gene transfer therapy in LGMD2D	DB, PC, randomized, efficacy	LGMD2D	Safety	Completed	< http://ClinicalTrials.gov/show/NCT00494195 >
MYO-029	DB, PC, randomized	BMD/FSH/LGMD	Safety	Completed	< http://ClinicalTrials.gov/show/NCT00104078 >
Deflazacort	DB, PC, randomized, efficacy	LGMD2B/ Miyoshi myopathy/ dysferlinopathy	Muscle strength	Completed	< http://ClinicalTrials.gov/show/NCT00527228 >
SomatoKine (Iplex) (rhIGF-I/rhIGFBP-3)	Non-randomized, OL	Myotonic dystrophy type 1	Safety	Completed	< http://ClinicalTrials.gov/show/NCT00233519 >
Dehydroepiandrosterone (DHEA)	DB, PC, randomized, efficacy	Myotonic dystrophy type 1	Muscle strength	Completed	< http://ClinicalTrials.gov/show/NCT00167609 >
Methylphenidate	DB, PC, randomized, efficacy	Myotonic dystrophy type 1	Changes in excessive daytime sleepiness	Completed	< http://ClinicalTrials.gov/show/NCT01421992 >
Mexiletine	DB, PC, randomized, efficacy	Myotonic dystrophy type 1	Safety, 6MWD	In progress	< http://ClinicalTrials.gov/show/NCT01406873 >

cTnI, cardiac troponin I; DB, double-blind; LGE, late gadolinium enhancement; MC, multicentre; MPI, myocardial perfusion index; 6MWD, 6-minute walking distance; NIRS, near-infrared spectroscopy; OL, open-label; PC, placebo-controlled; US, ultrasound.

Gene replacement therapies

Myoblast transfer

The interest in this field was initially derived from the observation that the injection of normal myoblasts into the muscle of dystrophic *mdx* mice rendered many of the fibres in the vicinity of the injection dystrophin-positive. Presumably, the normal myoblasts fused with the host muscle fibres. In the early 1990s, some investigators reported having obtained similar results in children with DMD. However, several carefully conducted and extensive studies carried out on a number of boys followed in different centres in the US and Canada have concluded that myoblast transplant is not clinically effective in DMD.

This is likely to be partly due to the immune rejection of donor myoblasts (usually from the father) so that, after a few months, there are no donor-derived dystrophin-positive fibres and virtually no donor-derived nuclei. Other major limitations of this approach would be the difficulties in obtaining cultured myoblasts in sufficient quantities for treatment and the problem of targeting multiple muscles, including the respiratory and cardiac muscles.

Whilst there does not appear to be any value in myoblast transfer, studies on bone marrow, mesoangioblasts, or muscle-derived stem cells have generated increased interest and enthusiasm.

Genetic therapies

Much has been written on the possibility of treating hereditary diseases by gene therapy, and various strategies are being considered using different delivery systems, including viral vectors (for example, DNA adenoviruses or RNA retroviruses) and direct gene transfer. The technical difficulty of each of these approaches is considerable and largely unresolved. In the case of DMD, problems are compounded by the immense size of the dystrophin gene that has to be transferred, limiting the choice of the viral vectors available.

A large number of experiments have been carried out in the *mdx* mice using an adenoviral vector, which has the advantage of being able to contain the entire cDNA of dystrophin. These studies have shown an excellent capability of transfecting individual muscles, following direct injection. However, there is significant immune reaction toward the viral vector itself. Moreover, and in common with all gene replacement approaches, there is also some concern for the immunogenicity of dystrophin per se, as this molecule will not be recognized as 'self' in DMD patients who have never produced dystrophin. The immune reaction to dystrophin and the viral vector can be overcome by the administration of immunosuppressive drugs. More encouraging results, in terms of viral immunogenicity, have been achieved by using the adeno-

associated virus (AAV), of which different serotypes exist. The AAV is non-pathogenic and is also very efficient in muscle transfection. One limitation is the inability of AAV to package the entire dystrophin cDNA. There are also some technical difficulties related to the production of large quantities of the virus for possible therapy in DMD. Regarding the first point, several authors have exploited the observation that some BMD patients have a mild phenotype, despite large in-frame deletions removing nearly 50% of the coding region. A mild BMD phenotype has been reported, for example, in patients with a deletion of exons 16–48, confirming that a large part of the rod domain appears dispensable, as long as the N- and C-termini of dystrophin are retained. In view of the limited immunogenicity of the AAV, this ‘mini-dystrophin gene’ appears a promising approach. A recent study from the group of Jerry Mendell at Columbus University in Ohio assessed the safety and efficacy of a single injection of an AAV vector containing a mini-dystrophin gene into a leg muscle in a group of six DMD boys (Mendell *et al.* 2010). Although the treatment was, on the whole, well tolerated, there was a robust immune reaction against the transgene which limited the efficacy. This is probably a combination of the immune reaction against the AAV vector, compounded by the choice of a promoter that was not selective for skeletal muscle. Current studies are concentrating on the use of muscle-specific promoters to limit the expression of the transgene in non-muscle tissue, hence blunting the possibility of an immune reaction, and on assessing in preclinical models the safety and feasibility of whole limb perfusion.

Other systems avoiding viral transfer have been also tried. Studies have been performed, using direct plasmid DNA injection in muscle. These studies followed from earlier observations that injection of plasmid DNA or RNA directly into mouse skeletal muscle can result in significant expression of reporter genes in muscle cells and no special delivery system is necessary. With regard to the transfer of the dystrophin gene, a human dystrophin plasmid cDNA is expressed in transfected cell cultures, as well as in *mdx* mouse muscle after being injected intramuscularly. Unfortunately, the efficiency of this approach remains too low for it to have any practical applications.

The possibility of dystrophin gene correction, using antisense oligonucleotides or with chimeric DNA/RNA oligonucleotides, is currently the most rapidly advancing approach. These approaches are usually referred to as ‘oligonucleotide-mediated gene therapy’. The most studied of these approaches is the use of antisense oligonucleotides targeted against acceptor or donor splice sites of the dystrophin gene. The idea is to modify the physiological splicing of the dystrophin gene and so create ‘functional in-frame deletions’ in patients with out-of-frame deletions or stop codons located in an exon. These

methods were initially developed in the *mdx* mouse, which carries a point mutation in exon 23. Various investigators have attempted to induce skipping of exon 23, which carries the deleterious mutation, and the creation of a functional in-frame deletion by targeting the donor or acceptor splice site of this exon. The results, initially *in vitro* (that is, in muscle cell cultures of the *mdx*), but more recently *in vivo*, have been very encouraging. This approach has also been applied to *mdx* cardiomyocyte cultures, although the efficiency appears to be lower, when compared to skeletal muscle.

A similar approach has been attempted in DMD patients carrying an exon 45 deletion, using an antisense oligoribonucleotide targeted against splicing enhancer sequences located in exon 46. Deletion of exon 45 is an out-of-frame deletion and usually results in DMD. Exon 46 contains a purine-rich sequence that has been shown to regulate the splicing of exon 46. These sequences have been named 'splicing enhancer sequences', and targeted antisense oligonucleotides against them have been shown to increase the splicing of exon 46. This eventually results in a 'functional larger deletion' of exons 45 and 46, with the restoration of the reading frame of dystrophin mRNA and dystrophin production (see Fig. 13.14). This approach was found to be successful in muscle cell cultures from a DMD patient. Other dystrophin exons carry apparent splicing enhancer sequences (for example, exons 43, 44, 46, 50, and 51).

A number of different exons are currently being targeted by antisense oligonucleotide approaches, and phase I, phase II, and phase III clinical trials, aiming at targeting exon 51, have been performed, including two studies using a

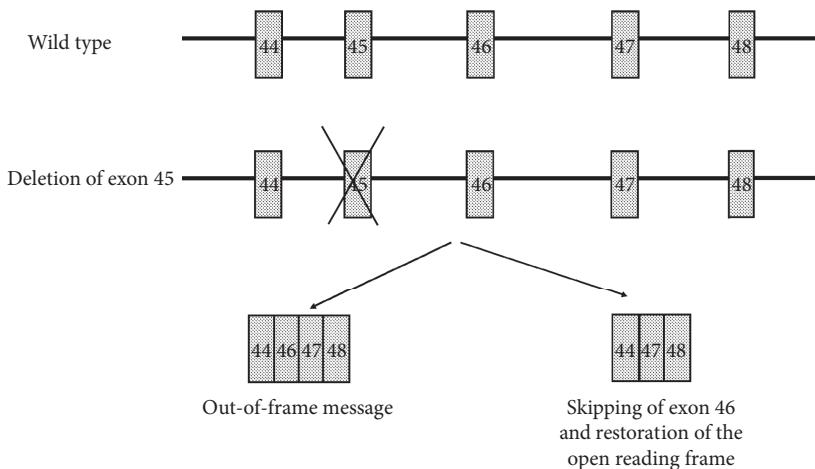


Fig. 13.14 Skipping of exon 46 and restoration of the ORF.

particular type of antisense oligonucleotide chemistry (morpholino) by one of the authors (Muntoni). These studies require systemic weekly administration of the antisense molecule and have demonstrated to induce the appropriate exon skipping and restoration of the dystrophin protein in DMD boys. Early indications appear to suggest also a clinical benefit in children recruited in both open-label extension and randomized, placebo-controlled studies. The precise extent of the long-term clinical benefit and long-term safety of these compounds are currently unknown. Nevertheless, the proof of concept, resulting from the positive outcome of the early exon 51 trials, has triggered a number of other studies targeting other exons, some already started (see Table 13.8 for further details). More information on exon skipping can be found at the following websites:

- ◆ <<http://www.mdex.org.uk/>>;
- ◆ <<http://www.humgen.nl/lab-aartsma-rus/index%20for%20parents.html>>;
- ◆ <<http://www.skip-nmd.eu/>>.

One of the obvious limitations of the antisense approach is the need for a repeated systemic delivery and the lack of effective cardiac targeting. The latter shortcoming is being addressed by next-generation chemistries currently under development (see <<http://www.mdex.org.uk/>>).

Stem cell therapy

Stem cells with the potential of developing into muscle cells can be obtained from bone marrow, umbilical blood, or even the early embryo. There is a possibility that such stem cells might prove to be another approach to the treatment of DMD. For example, bone marrow (haemopoietic) stem cells from normal mice have been shown to relocate in the muscle of *mdx* mice and produce dystrophin. Unfortunately, in these experiments, the proportion of muscle cells producing dystrophin was very small. Nevertheless, these observations do raise the possibility of the therapeutic potential of using some form of stem cells for DMD. One of the most promising stem cells for systemic delivery is the mesoangioblast. Experimental studies in dystrophic dogs receiving the mesoangioblasts have been encouraging, and this led to a phase I clinical trial in a small group of DMD boys, currently under investigation.

Upregulation of other genes/proteins

The observation that utrophin, a homologue of dystrophin, can partially compensate for the absence of dystrophin, once upregulated, in the *mdx* mice has generated a lot of interest in this as a potential treatment strategy for DMD. The idea is to identify drugs that could significantly upregulate the production of

utrophin in skeletal muscle. This protein is expressed at the sarcolemma during fetal life, but only at the neuromuscular junction in adult muscle. It is believed that the upregulation of its production at the sarcolemma in DMD might compensate for the lack of dystrophin. Another advantage of utrophin is the likely absence of any immune reaction to this protein, as it should be recognized as 'self'. Indeed, the re-introduction of dystrophin, following gene therapy, in the *mdx* mice is followed by a strong immune reaction against dystrophin. This does not occur with utrophin, since it is physiologically produced in all individuals with DMD.

Studies have also shown that the upregulation of integrin $\alpha 7$ (see Fig. 10.1) is capable of preventing the development of pathology in the *mdx* mice. Thus, the possibility that some pharmacological agent will be found that will upregulate utrophin, integrin $\alpha 7$, or even some other protein, and ultimately prove therapeutic in DMD is an attractive one (see Table 13.8).

Summary and conclusions

Although DMD is not curable, effective treatments are available that can improve the quality of life and survival of affected boys. Paramount is the maintenance of good general health, with an emphasis on good nutrition and weight control, the prevention of deformities, and the preservation of respiratory function. Corticosteroids can prolong walking and preserve respiratory function, but the side effects need to be actively managed. The development of deformities can be delayed by passive exercises, and ambulation can be prolonged with KAFOs and various other orthoses. Scoliosis is a particularly serious problem, because the progressive thoracic deformity restricts adequate pulmonary ventilation and aggravates respiratory problems. Spinal surgery is a major operation that carries its own complications, but patients with progressive scoliosis should be considered for this operation, although corticosteroid administration has reduced the need for this operation. Whilst occasionally patients may develop rhabdomyolysis following a general anaesthetic, this appears to be an infrequent complication, almost always restricted to younger children, and can be prevented by avoiding certain muscle relaxants. Whilst, for the majority of patients, there are no serious anaesthetic or post-operative problems, the anaesthetist and surgeon need to be prepared against these eventualities, and careful monitoring of respiratory and cardiac function should be performed before and after any major surgery.

Impaired pulmonary function is a major factor in morbidity and mortality. Over 90% of deaths are due to pulmonary infection and respiratory failure. All respiratory infections must be treated vigorously. In the later stages of the

disease, assisted ventilation can be helpful in relieving symptoms associated with hypoventilation and prolonging life.

The psychological effects of the disease on the patient himself, as well as on his parents and unaffected sibs, cannot be ignored. Open and frequent discussions between all those concerned, including health care professionals, should be encouraged. The educational needs of affected boys can also raise problems for those who are severely handicapped and may require special care. There are increasing opportunities for those who are highly intelligent to achieve well in higher education, despite their severe physical handicap.

Whilst stem cell and gene therapy approaches are progressing, but at a slow pace due to the formidable task of targeting skeletal muscle, the most abundant tissue in our body, genetic therapy trials using antisense oligonucleotides have very rapidly developed, and currently multiple trials are under way, and the first positive results from randomized, placebo-controlled studies have been announced. This is quite exciting, as this would represent the first specific therapeutic approach potentially capable of altering the disease course in DMD.

Whilst there are now good reasons to entertain cautious optimism that an effective treatment for at least some of the DMD boys might have been found, nevertheless this approach does not represent a definitive curative approach, so efforts are continuing to target different disease mechanisms at a pace that could not be anticipated until a few years ago, as summarized in Table 13.8.

Because of the growing awareness of the importance of physiotherapy in muscular dystrophy, an international congress on the subject was held in Italy in 1984, the proceedings of which have now been published in **detail** (*Cardiomyology*, Vol. 3, nos. 2–3, 1984). The reader will find therein helpful information, including some useful guidelines proposed by the European Alliance of Muscular Dystrophy Associations.

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Appendix 1

Egen Klassifikation Scale
Version 2 (EK2)

Name

DOB

Date of assessment

Date of spinal surgery

Assessor

NOTE: * Score the best you have done in the last two weeks, especially if there is variation between good and bad days

(please circle)

1	Ability to use wheelchair. How do you get around indoors and outdoors?	N/A
	Able to use a manual wheelchair on flat ground, 10 m < 1 minute	0
	Able to use a manual wheelchair on flat ground, 10 m > 1 minute	1
	Unable to use manual wheelchair, requires power wheelchair	2
	Uses power wheelchair, but occasionally has difficulty steering	3
2	Ability to transfer from wheelchair. How do you transfer from your wheelchair to a bed?	N/A
	Able to transfer from wheelchair without help	0
	Able to transfer independently from wheelchair, with use of aid	1
	Needs assistance to transfer with or without additional aids (hoist, easy glide)	2
	Needs to be lifted with support of head when transferring from wheelchair	3
3	Ability to stand. Do you sometimes stand? How do you do this?	N/A
	Able to stand with knees supported, as when using braces	0
	Able to stand with knees and hips supported, as when using standing aids	1
	Able to stand with full body support	2
	Unable to be stood	3

4	Ability to balance in the wheelchair. Can you bend forwards and to the sides and return to the upright position?	N/A
	Able to push himself upright from complete forward flexion by pushing up with hands	0
	Able to move the upper part of the body >30° in all directions from the upright position, but cannot push himself upright as above	1
	Able to move the upper part of the body <30° from one side to the other	2
	Unable to change position of the upper part of the body, cannot sit without total support of the trunk and head	3
5	Ability to move the arms. Can you move your fingers, hands, and arms against gravity?	N/A
	Able to raise the arms above the head with or without compensatory movements	0
	Unable to lift the arms above the head, but able to raise the forearms against gravity, i.e. hand to mouth with/without elbow support	1
	Unable to lift the forearms against gravity, but able to use the hands against gravity when the forearm is supported	2
	Unable to move the hands against gravity, but able to use the fingers	3
6	Ability to use the hands and arms for eating. Can you describe how you eat?	N/A
	Able to eat and drink without elbow support	0
	Eats or drinks with support at elbow	1
	Eats and drinks with elbow support; with reinforcement of the opposite hand with or without aids	2
	Has to be fed	3
7	Ability to turn in bed. How do you turn in bed during the night?	N/A
	Able to turn himself in bed with bedclothes	0
	Needs some help to turn in bed or can turn in some directions	1
	Unable to turn himself in bed. Has to be turned 0–3 times during the night	2
	Unable to turn himself in bed. Has to be turned >4 times during the night	3
8	Ability to cough. How do you cough when you have to?	N/A
	Able to cough effectively	0
	Has difficulty to cough and sometimes needs manual reinforcement. Able to clear throat	1

8 Ability to cough. How do you cough when you have to?	N/A
Always needs help with coughing. Only possible to cough in certain positions	2
Unable to cough. Needs suction and/or hyperventilation techniques or IPPB in order to keep airways clear	3
9 Ability to speak. Can you speak so that what you say can be understood if you sit at the back of a large room?	N/A
Powerful speech. Able to sing and speak loudly	0
Speaks normally but cannot raise his voice	1
Speaks with quiet voice and needs a breath after 3–5 words	2
Speech is difficult to understand, except to close relatives	3
10 Physical well-being (this relates to respiratory insufficiency only—see manual)	N/A
No complaints, feels good	0
Easily tires. Has difficulty resting in a chair or in bed	1
Has loss of weight, loss of appetite, scared of falling asleep at night, sleeps badly	2
Experience additional symptoms: change of mood, stomach ache, palpitations, perspiring	3

Additional items

11 Daytime fatigue. Do you have to organize your day or take a rest to avoid getting too tired?	N/A
Does not get tired during the day	0
Need to limit activity to avoid getting too tired	1
Need to limit my activity and have a rest period to avoid getting too tired	2
Get tired during the day, even if I rest and limit activity	3
12 Head control. How much head support do you need in your wheelchair?	N/A
Does not need head support	0
Needs head support when going up and down slope (15° standard ramp)	1
Needs head support when driving wheelchair	2
When sitting still in a wheelchair, needs head support	3

13 Ability to control joystick. What kind of joystick do you use to control your chair?	N/A
Uses a standard joystick without special adaptation	0
Uses an adapted joystick or has adjusted wheelchair in order to use joystick	1
Uses other techniques for steering than joystick such as blowing sucking systems or scanned driving	2
Unable to operate wheelchair. Needs another person to operate it	3
14 Food textures. Do you have to modify your food in any way in order to eat it?	N/A
Eats all textures of food	0
Eats cut-up/chunky food or avoids hard/chewy foods	1
Eats minced/puréed food, with supplementation, as required	2
Main intake consists of being tube-fed	3
15 Eating a meal (with or without assistance). How long does it take to complete a meal?	N/A
Able to consume a whole meal in the same time as others sharing the meal	0
Able to consume a whole meal in the same time as others, only with encouragement, or needs some additional time (approximately 10 minutes)	1
Able to consume a whole meal but requires substantially more time, compared to others, eating the same meal (15 minutes or more extra)	2
Unable to consume a whole meal	3
16 Swallowing. Do you ever have problems with swallowing?	N/A
Never has problems when swallowing and never chokes on food/drink	0
May experience occasional (less than once a month) problems swallowing certain types of food or occasionally chokes	1
Has regular trouble swallowing food/drink or chokes on food/drink (more than once a month)	2
Has trouble swallowing saliva or secretions	3
17 Hand function. Which of these activities can you do?	N/A
Can unscrew the lid of a water or fizzy drink bottle and break the seal	0
Can write two lines or use computer keyboard	1
Can write signature or send text or use remote control	2
Cannot use hands	3

TOTAL SCORE /51

Comments: any comments, including lack of clarity, difficulty scoring, etc. For example, relating to age—reasons any items were not applicable (N/A) or swallowing—perhaps reasons swallowing was a problem or when or how often.

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Appendix 2

The North Star Ambulatory Assessment

We have attempted to give clear explanations of the methods employed to achieve motor goals, but it is not possible to be exhaustive in the descriptions, particularly of modifications to activity. Whilst DMD children may generally present with recognizable adaptations to activity due to the underlying progressive muscular weakness, they may modify their activity to achieve functional goals in slightly differing ways. Generally, activities are graded in the following manner:

- 2: 'Normal' — no obvious modification of activity
- 1: Modified method but achieves goal independent of physical assistance from another
- 0: Unable to achieve independently

Gowers' manoeuvre

Definition of Gowers' manoeuvre

The child turns towards the floor (generally into a four-point kneeling position) to place hands on the floor to assist rising, walks hands back in towards him then uses arms to 'climb' up legs to achieve upright standing. A wide base of support is often assumed through the phases of rising from the floor. See Fig. A2.1.

Stair Climb

As it is not possible to ensure standardisation, or availability, of flights of stairs, we are asking that a box step (approximately 15cm high) is used to assess single step climb and descend. A plinth or other immovable object may need to be available to provide support.

The following tables give test details and instructions for the patient and a scoring sheet with details for grading. They should be used in conjunction. Please familiarize yourself with the test detail before starting to evaluate patients.

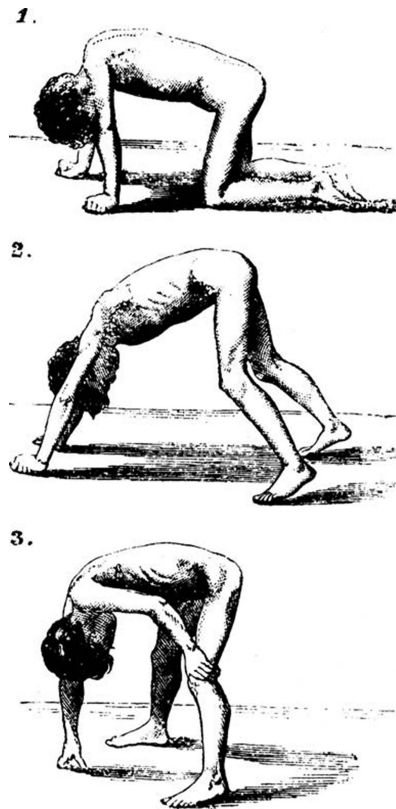


Fig. A2.1 Gowers' manoeuvre.

From W. R. Gowers' Pseudo-hypertrophic muscular paralysis, 1879.

Test detail and instructions to patient

Activity	Instructions to patient	Start position/test detail	Comments
1. Stand	Can you stand up tall for me for as long as you can and as still as you can	Feet should be close together and heels on the ground if possible. Arms by sides. NO shoes should be worn	Best done on the floor rather than on a mat. Whichever is chosen maintain consistency through repeated testing sessions. Minimum count of 3 seconds to score 2
2. Walk	Can you walk from A to B (state to and where from) for me	Walk without shoes/socks on. Should be enough of a distance to observe 'normal gait' for that subject	A value judgement needs to be made in scoring— if the patient generally toe walks but occasionally gets heels flat, or can on request but doesn't usually, they should score 1

Activity	Instructions to patient	Start position/test detail	Comments
3. Stand up from chair	Stand up from the chair keeping your arms folded if you can	Starting position 90° hips and knees, feet on floor/supported on a box step.	A size-appropriate chair or height adjustable plinth should be used. Arms should be kept crossed throughout the activity to score 2
4. Stand on one leg—right	Can you stand on your right leg for as long as you can?	Minimum count of 3 seconds to score 2. NO shoes should be worn.	Best done on the floor rather than on a mat. Whichever is chosen maintain consistency through repeated testing sessions
5. Stand on one leg—left	Can you stand on your left leg for as long as you can?	Minimum count of 3 seconds to score 2. NO shoes should be worn.	Best done on the floor rather than on a mat. Whichever is chosen maintain consistency through repeated testing sessions
6. Climb box step—right	Can you step onto the top of the box using your right leg first?	Stands facing the box step. Step should be approximately 15cm high	Support may be provided by the use of a height adjustable plinth, or, if not available a 'neutral' hand from the therapist.
7. Climb box step—left	Can you step onto the top of the box using your left leg first?	Stands facing the box step. Step should be approximately 15cm high	Support may be provided by the use of a height adjustable plinth, or, if not available a 'neutral' hand from the therapist
8. Descend box step—right	Can you step down from the box using your right leg first?	Stands on top of the box step facing forwards. Step should be approximately 15cm high	Support may be provided by the use of a height adjustable plinth, or, if not available a 'neutral' hand from the therapist
9. Descend box step—left	Can you step down from the box using your left leg first?	Stands on top of the box step facing forwards. Step should be approximately 15cm high	Support may be provided by the use of a height adjustable plinth, or, if not available a 'neutral' hand from the therapist

Activity	Instructions to patient	Start position/test detail	Comments
10. Gets to sitting	Can you get from lying to sitting?	Starting position supine on a mat. No pillow should be used under head	If patient turns into prone or towards the floor to work their way into sitting 1 should be scored
11. Rise from floor	Get up from the floor using as little support as possible and as fast as you can (from supine)	Starting position supine with arms by sides, legs straight. No pillow to be used	Activity should be attempted without use of furniture in the first instance. Do not note time if a chair has to be used
12. Lifts head	Lift your head to look at your toes keeping your arms folded	Supine on a mat. No pillow should be used	Ask patient to keep arms crossed over chest during the activity to avoid self-assist. Also ask to look at toes to ensure neck is flexed—should be a chin to chest manoeuvre
13. Stands on heels	Can you stand on your heels?	Standing on the floor. No shoes to be worn	Watch for inversion. If substantial inversion but forefeet are still lifted—score 1. If only inversion with lateral border of foot still on the ground score 0
14. Jump	How high can you jump?	Standing on the floor, feet fairly close together	Want height, not forward movement. Small amount of forward movement acceptable
15. Hop right leg	Can you hop on your right leg?	Starting position standing on floor on right leg. No shoes should be worn	Needs obvious floor clearance to score 2
16. Hop left leg	Can you hop on your left leg?	Starting position standing on floor on right leg. No shoes should be worn	Needs obvious floor clearance to score 2

Activity	Instructions to patient	Start position/test detail	Comments
17. Run (10m)	Run as fast you can to (give point)	A straight 10m walkway should be clearly marked in a quiet department or corridor. A stopwatch should be used to time the walk. Be consistent as to whether shoes are worn or not. Ensure safety of patient. They should self select speed after being asked to go 'as fast as they can'	'Duchenne jog'— not a true run (there probably IS a double support phase), but more than a walk. Typically characterized by excessive use of arms, trunk rotation, substantial 'waddle'. No real 'pushoff'

North Star Ambulatory Assessment—Score Sheet

Activity	2	1	0	Comments
1. Stand	Stands upright, still and symmetrically, without compensation (with heels flat and legs in neutral) for minimum count of 3 seconds	Stands still but with some degree of compensation (e.g. on toes or with legs abducted or with bottom stuck out) for minimum count of 3 seconds	Cannot stand still or independently, needs support (even minimal)	
2. Walk	Walks with heel-toe or flat-footed gait pattern	Persistent or habitual toe walker, unable to heel-toe consistently	Loss of independent ambulation—may use KAFOs or walk short distances with assistance	
3. Stand up from chair	Keeping arms folded Starting position 90° hips and knees, feet on floor/supported on a box step	With help from thighs or push on chair or prone turn	Unable	
4. Stand on one leg—right	Able to stand in a relaxed manner (no fixation) for count of 3 seconds	Stands but either momentarily or needs a lot of fixation e.g. by knees tightly adducted or other trick	Unable	

Activity	2	1	0	Comments
5. Stand on one leg—left	Able to stand in a relaxed manner (no fixation) for count of 3 seconds	Stands but either momentarily or needs a lot of fixation e.g. by knees tightly adducted or other trick	Unable	
6. Climb box step—right	Faces step—no support needed	Goes up sideways or needs support	Unable	
7. Climb box step—left	Faces step—no support needed	Goes up sideways or needs support	Unable	
8. Descend box step—right	Faces forward, climbs down controlling weight bearing leg. No support needed	Sideways, skips down or needs support	Unable	
9. Descend box step—left	Faces forward, climbs down controlling weight bearing leg. No support needed	Sideways, skips down or needs support	Unable	
10. Gets to sitting	Starts in supine—may use one hand to assist	Self assistance e.g.— pulls on legs or uses head-on-hands or head flexed to floor	Unable	
11. Rise from floor	From supine—no evidence of Gowers' manoeuvre	Gowers' evident	(a) NEEDS to use external support object e.g. chair OR (b) Unable	Time (00.0s)
12. Lifts head	In supine, head must be lifted in mid-line. Chin moves towards chest	Head is lifted but through side flexion or with no neck flexion	Unable	
13. Stands on heels	Both feet at the same time, clearly standing on heels only (acceptable to move a few steps to keep balance) for count of 3	Flexes hip and only raises forefoot	Unable	

Activity	2	1	0	Comments
14. Jump	Both feet at the same time, clear the ground simultaneously	One foot after the other (skip)	Unable	
15. Hop right leg	Clears forefoot and heel off floor	Able bend knee and raise heel, no floor clearance	Unable	
16. Hop left leg	Clears forefoot and heel off floor	Able bend knee and raise heel, no floor clearance	Unable	
17. Run (10m)	Both feet off the ground (no double stance phase during running)	'Duchenne jog'	Walk	Time (00.0s)
				TOTAL = /34

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Appendix 3

Muscular Dystrophy Associations and Groups in Various Countries (2013)

Argentina

CIDM
Zapiola 740 CP 1426
Buenos Aires
Association Distrofia Muscular
Cordoba 5428, BUENOS AIRES

Asociacion Distrofia Muscular
Para las Enfermedades
Neuromusculares
Avda. Rivadavia 4951 PB Dto.2
C1424CEE Capital Federal
Argentina
Tel: +54 11 15-3-593-9265
Email: admargentina@gmail.com
Website: www.adm.og.ar

Armenia

Charity Association of
Neurohereditary Diseases
Amirya str.10. Apt 15, Yearevan
3750
Republik of Armenia
Tel: 374 1 56 17 81
Fax: 374 1 52 59 19
Email: alamat@arminco.com

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Muscular Dystrophy Association of
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PO Box 24
Torrensville Plaza
SA 5031
Tel: 61 8 8234 5266
Fax: 61 8 8234 5866
Email: info@mdsa.org.au
Webmail: www.mdasa.org.au

Muscular Dystrophy Association of
NSW
Suite 101, 5 Bay Drive
Meadowbank 2114
Postal:
PO Box 1365
Meadowbank 2114
Freecall: 1800 635 109
Tel: (02) 9809 2111
Fax: (02) 9809 4177
Email: info@mdnsw.org.au
Website: www.mdnsw.org.au

Muscular Dystrophy Association of
Queensland
Unit 13,
191 Hedley Ave
Hendra Qld 4011
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Locked Bag 3000

Eagle Farm BC Qld 4009
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Fax: (07) 3607 1899
Email: info@mdqld.org.au
Website: www.mdqld.org.au

Muscular Dystrophy Association Inc
111 Boundary Road
North Melbourne
Victoria 3051
Postal:
PO Box 1050
Moonee Ponds
Victoria 3039
Tel: +61 3 9320 9555
Fax: +61 3 9320 9595
Email: bms@mda.org.au
Website: www.mda.org.au

Muscular Dystrophy Association of
Tasmania
26 Goulburn Street
Hobart
Tasmania 7000
Tel: 03 6231 3273
Email: ashfords@dodo.com.au
Website: www.mdtasmania.org.au

Muscular Dystrophy Association of
ACT
Unit 5
48 Brookes Street
Mitchell ACT 2911
Tel: (02) 6241 1220
Fax: (02) 6241 1224
Email: info@nican.com.au
Website: www.nican.com.au

Muscular Dystrophy Research
Association of
Western Australia

Suite B The Niche
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Nedlands
WA 6009
Postal:
PO Box 680
Nedlands WA 6909
Tel: 08 9380 3400
Fax: 08 9382 2067
Email: info@mdwa.org.au
Website: www.mdwa.org.au

Austria

Österreichische Gesellschaft zur
Bekämpfung der
Muskelrankheiten
Währinger Gürtel 18-20
Postfach 23
A-10097 Wien
Tel: +431 404003112
Email: muskelgesellschaft@gmx.at
Website: www.members.aon.at/muskel/

Support group for Muscular
Dystrophy - Upper Austria
c / o Bayer Wilfried
Altensam 49
4800 Attnang - Puchheim
Phone: 0 76 74/6 6444
E-mail: info@muskelkrank.at

Austrian Society for Muscular
Dystrophy
National Group Vienna
Währingergürtel 18-20
1097 Vienna
Tel: 01/40400 3112
Fax: 01/3141 40400
E-mail: muskelgesellschaft@gmx.at

Austrian Society for Muscular
Dystrophy
National Group Styria
Secretary: Dr. Barbara Streitfeld
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8053 Graz
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Fax: 0 316/26 10 94
E-mail: office@muskelkranke-stmk.at

Belgium

Vlaamse Vereniging Neuro-
Musculaire
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Website: www.nema.be

Association Belge contre les
Maladies
Neuromusculaires (ABMM)
Rue Archille Chavee 52/02
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Belgique
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Website: www.abmm.be

Brazil

Associao Brasileria de Distrofia
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715 Butantã CEP:05505-030
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Email: secretaria@abdim.org.br
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canada

Muscular Dystrophy Canada
Montreal
1425
Rene-Levesque Blvd. West,
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Montreal
QC H3G 1T7
Tel: 514 393 3522

Email: infoquebec@muscle.ca
 Website: www.muscle.ca/quebec

Muscular Dystrophy Canada
 Atlantic
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 Dartmouth
 Nova Scotia B3B 0G1
 Tel: 902 429 6322
 Email: infoatlantic@muscle.ca
 Website: www.muscle.ca/atlantic-canada

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 1000 ZAGREB,
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Cyprus

Muscular Dystrophy Association of
 Cyprus
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 Dhometios
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 Nicosia CY 1638
 Email: mad@cing.ac.cy
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Czech Republic

Asociace Muskularinich Dystrofiku
 V CR
 Petyrkova 1953/24
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 Tel: 420 7 9337 77

Denmark

Muskelsvindfonden
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 http: www.muskelsvindfonden.dk

EAMDA (Secretariat)
 European Alliance of Muscular
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 Boris Stustarsic EAMDA President
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info@eamda.eu
 Website: www.eamda.eu

The EAMDA Secretariat can supply
 updated listings of all European
 member associations along with their
 telephone, fax and emails.

ENMC
 European Neuromuscular
 Centre
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 The Netherlands
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Egypt

Egypt Muscular Dystrophy
 Association
 19 Elmesafer Khana Street
 Elgomrok, Alexandria 21111

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Eesti Lihasehaigete Selts
(The Estonian Association of
Muscular Disorders)
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11317 Tallinn
Email: els@els.ee
Website: www.else.ee

Finland

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France

Association Francaise contre les
Myopathies (AFM)
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Email: afm@mail.afm.genethon.fr
Website: www.afm.telethon.com

Germany

Deutsche Gesellschaft fur
Mukelranke e V (DGM)
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Honduras

British Honduras Muscular
Dystrophy Association
Insituto Hondureno do
Rehabilitacion
Tegucigalpa Municipio del
Distrito Central Honduras

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Hungaria Neuromuscular
Disorders Association
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Indian Association of
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Israel Society for Neuromuscular
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Tokyo 162-0051
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Website: www.jmda.or.jp

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Ldelaguila@conadisperu.gob.pe

Website: www.usuarios.discapnet.es/adm_peru

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Hospital de Santa Maria
Centro Estudos Egas Moiz
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The Interregional Association of
Assistance to People
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Website: www.omdvsr.sk

Slovenia

Drustvo Misicno obsoleih
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Website: www.drustvo-distrofikov.si

South Africa

Muscular Dystrophy
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Muscular Dystrophy
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Sweden

Neurologiskt Handikappades
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Riksförbundet för
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Switzerland

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Website: www.muskelkrank.ch

Association Suisse Romande contre
Et Italienne contre les Myopathie
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1400 Yverdon
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Email: info@asrim.ch
Website: www.asrim.ch

Taiwan

Taiwan Muscular Dystrophy
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Sanming Area
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Website: www.mda.org.tw

Turkey

Türkiye Kas Hastalıkları Derneği
Yesilköy Hatboyu Cad.No.12,
34800 TR-Istanbul
Website: www.kasder.org.tr

United Kingdom

Muscular Dystrophy Campaign
(MDC)
61A Great Suffolk St.
London
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Tel: 020 7803 4800

Email: info@muscular-dystrophy.org

Website: www.muscular-dystrophy.org

Motor Neurone Disease Association

PO Box 246

Northampton

NN1 2PR

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Email: enquiries@mndassociation.org

Website: www.mndassociation.org

Ukraine

Ukrainian Muscular Dystrophy
Association (ERB)

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Lviv 39 79039

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Email: erb@lviv.net

Website: www.erb.com.ua

United States of America

Muscular Dystrophy Association of
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3300 East Sunrise Drive

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Email: mda@mdusa.org

Website: www.mda.org

Uruguay

Asociacion Uruguaya de

Aldeas Infantiles SOS

Silcinesa 3030

Montevideo

WAMDA

World Alliance of Muscular

Dystrophy Associations

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Yugoslavia, Federal Republic

Savez Distroficara

Jugoslavije

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and savez@distrofija.rs

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Zimbabwe

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Of Zimbabwe

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