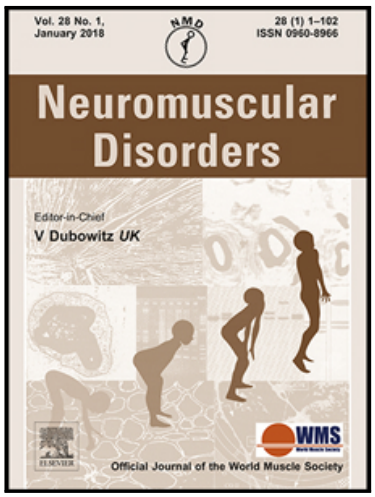

263rd ENMC International Workshop: Focus on female carriers of dystrophinopathy: refining recommendations for prevention, diagnosis, surveillance, and treatment. (June '21-May '22)

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Highlights

- *DMD* female carriers are a complex clinical and still neglected patient group
- Greater clinical and research focus on DMD female carriers is needed
- Minimum care standard for *DMD* female carriers should include genetic testing

- Preventive and therapeutic approaches can improve patient outcomes
- Patient registries are required for natural history studies and clinical trials

Keywords: dystrophinopathy; carrier; females; genetics, cardiomyopathy; high CK

1.0 Introduction

The *263rd ENMC International Workshop* was convened in Amsterdam 13th-15th May 2022. This was a hybrid meeting (participants were either face to face or virtual via an on-line link) and it followed two virtual preparatory sessions in June and November 2021 (see preparatory activities in https://www.enmc.org/download/7849/) [1]. The meeting brought together 19 participants from 7 countries in Europe and the USA from backgrounds including: basic science, genetics, neuromuscular disease, cardiology, patient representatives and there were three trainees/young participants supported by the ENMC *Young Scientist Programme*. The aims of the workshop were to explore and discuss the definition of '*DMD*-carriers' (including genetic profile), diagnostic pathways, clinical features, prevention, and potential therapies. The workshop also reviewed the implications for medical care with particular emphasis on cardiac surveillance. In this manuscript, we will use the terminology '*DMD*-carriers' for females who carry pathogenic variants in the *DMD* gene, independently of whether the variant is associated with Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD).

Most females who carry pathogenic variants in the *DMD* gene do not manifest any symptoms and no abnormalities are identified on examination, cardiac testing, or laboratory investigations, including blood and muscle biopsy analysis. However, some females have or develop: hyperCKaemia [persistently raised blood creatine kinase (CK) levels without symptoms or signs], cardiomyopathy, muscle symptoms (myalgia with or without progressive weakness) which can significantly impact their quality of life. Cognitive impairment may also occur due to the abnormal *DMD* gene, although large cohort studies on cognitive features in *DMD*-carriers are still lacking [2].

Thus, given the diversity in clinical features of *DMD*-carriers, this workshop aimed to:

(i) review what is already known about the consequences of pathogenic variants in the *DMD* gene in females, their identification, and explore the risk of disease recurrence, disease prevention, and family planning;

(ii) discuss and find consensus on the definition of the term '*DMD*-carriers';

(iii) define the care-needs of females who develop clinical effects, including their access to novel therapies;

(iv) propose consensus recommendations for appropriate surveillance including a holistic and systematic approach to the management of this neglected population.

2.0 Clinical and pathological aspects

2.1 What services do *DMD*-carriers themselves want and expect?

Elizabeth Vroom (EV) presented the results of a World Duchenne Organisation questionnaire with responses from respondents in 20 countries. Key patient expectations were for regular physical check-ups (77.6%), with a similar proportion wanting psychosocial support and counselling. However, physical review was only reported to be available in their country by 33.6% and psychosocial support by 29.6% of respondents. Regular cardiac assessments are recommended by the *International Standards of Care* DMD (2018), but only reported to be available routinely in 33.3% of countries. There were wide differences within and between countries about which services were available for these females, that could not be explained by the wealth of the country alone. Respondents also much wanted earlier testing of sisters of DMD boys than currently practiced (above 16 years of age), and for affected females to have access to psychosocial support regardless of whether they had an affected son. Carrier females want to be assessed and managed rather than in the context of being mother of a DMD son. The findings suggested that the health services available to *DMD*-carriers in most countries are poorly organised and patchy at best. Furthermore, when services are available, most females don't know how to access them.

From a genetic perspective, the diagnosis of *DMD*-carrier is clear-cut. However, medical insurance companies can vary in how they interpret this designation and its implications. Nicol Voermans (NV)

highlighted how the lack of distinction between different *DMD*-carrier phenotypes also impacts the way *DMD*-carriers are perceived by clinicians which, in turn, affects how they are subsequently investigated and treated. Patients commonly report encountering clinicians who do not know that the *DMD*-carrier status can result in various clinical phenotypes. She suggested that females presenting abnormal test results [e.g.: persistent elevated CK levels or left ventricular (LV) dysfunction], muscle symptoms and signs of weakness, should be categorised as 'patients with dystrophinopathy' and not just as '*DMD*-carriers'. Most carriers report always being poor at sports. This history should prompt CK measurements and, if an initial resting measure is indeterminate, it should be repeated after exercise to increase test sensitivity. Earlier genetic diagnosis would enable girls with cognitive impairment to access timely and appropriate educational support.

2.2 Experience from other X-linked neuromuscular disorders: XL-MTM1

NV presented the results of studies on carriers of X-linked myotubular myopathy (XL-MTM), as examples of how the spectrum of that phenotype can be determined by more inclusive recruitment of participants. Two large case series on XL-MTM carriers had been published, both with selection bias towards those who are more severely affected [3,4]. At the 2019 Family conference of the *ZusammenStark* patients' organisation, questions arose on the prevalence of symptomatic carriers and plans were made to determine the proportion of carriers of that condition who manifest clinical features [5].

An on-line cross-sectional questionnaire was first conducted among XL-MTM carriers. Participants were recruited with the help of patient associations, medical centres, and registries in the UK, Germany, and the Netherlands. The prevalence of manifesting carriers was found to be 51% (n = 76), subdivided into severe (wheelchair dependent, 3%), moderate (assisted ambulation, 9%) and mild (independent ambulation, 39%) phenotypes. In addition to muscle weakness (which was often asymmetrical), manifesting carriers frequently reported fatigue (70%) and exercise intolerance (49%) [6]. A nationwide cross-sectional study was then performed in an unselected Dutch cohort. We invited all females we could trace in the Netherlands, independent of previous hospital visits / neurological consultations so differently from what previously described.

Twenty-one carriers were included, of whom eleven (52%) were classified as manifesting, severe (n=2), moderate (n=2), mild (n=3) and minimal (only facial muscle weakness, n=4) phenotypes. Ten participants (48%) were classified as non-manifesting. Manifesting carriers had lower scores on 6-minute walk test and timed up-and-go test, compared to non-manifesting carriers [5].

These studies showed that approximately half of an unselected group of XL-MTM carriers have muscle weakness, which is often asymmetrical (three of whom previously classified as non-manifesting). The results of these two observational studies demonstrated variations in the severity of XL-MTM carriers and provide information to aid the design of clinical trials. They also show the value of harnessing data, only accessible through patient organizations, in resolving important clinical questions. A similar methodology could be used to characterise the *DMD*–carrier population [3,4].

2.2 Findings of the Danish '*DMD*-carrier' cohort study

John Vissing (JV) reported findings of a study of 53 females carrying pathogenic variants associated with DMD (N=33) or BMD (N=20), who underwent assessment comprising physical examination, dynamometry, muscle MRI, and cardiac evaluation – including cardiac MRI. Three females were asymptomatic at time of the study [7,8].

Muscle strength was reduced in a symmetric pattern of weakness similar to that observed in males with DMD or BMD. Muscle MRI showed increased fat fraction, often with striking asymmetry, in the posterior thigh compartment and calf muscles in two-thirds of the carriers studied. MRI-abnormalities correlated with reduced strength measurements on dynamometry. Overall, there were no major differences between the findings in females with BMD or DMD –pathogenic variants. These findings support use of muscle MRI to confirm muscle involvement in *DMD*-carriers [7].

2.3 Muscle pathology in *DMD*-carriers: the utility of a muscle biopsy

Rahul Phadke (RP) presented a literature review of the role of muscle biopsies in assessing females with molecularly confirmed or suspected dystrophinopathy. In general, muscle biopsies from asymptomatic dystrophinopathy carriers show minimal changes (mild variation in fibre size/fibre type) and mild abnormalities in dystrophin expression with weak or variable, discontinuous labelling at the myofiber sarcolemma, and reduced abundance on immunoblots. Symptomatic carrier biopsies show greater

variability, ranging from mild myopathic to florid dystrophic changes of Duchenne-like severity - including fibrosis, fatty infiltration necrosis and regeneration. Dystrophin-negative fibres are rarely observed [9]. A true mosaic immunostaining pattern of randomly dispersed dystrophin-negative and dystrophin-positive fibres is often seen in symptomatic carrier biopsies with reduced abundance and/or altered molecular weight on immunoblots. In a minority of carriers (usually asymptomatic) CK levels are normal but abnormalities in dystrophin expression are detectable in biopsies [10]. Utrophin is upregulated at the sarcolemma of both dystrophin-positive and dystrophin-deficient fibres. Most studies in the literature report a lack of clear association between the extent of skewed XCI, clinical phenotype, severity of myopathic/dystrophic changes and abnormalities of dystrophin expression in muscle biopsies [11]. Possible explanations are ascertainment bias, tissue-specific differences in XCI (blood vs skeletal muscle), sampling bias (needle vs open biopsy) due to patchy or uneven dystrophin expression in different areas of the same muscle, significantly different levels of dystrophin expression and XCI pattern between different muscles [12], and biochemical and genetic normalisation in multinucleate syncytial muscle fibres with increasing age [13]. RP concluded that applying new techniques such as digital immunofluorescent analysis of dystrophin and capillary Western immunoassay (Wes) for precise dystrophin quantitation across multi-centre cohorts of carrier biopsies would help in addressing a possible relationship between the amount of dystrophin and clinical phenotype.

3.0 Genetic diagnosis and X-chromosome inactivation

3.1 DMD gene mutation detection strategies: pre-conception, pre-natal and pre-implant testing

Alessandra Ferlini (AF) discussed known differences in *DMD* genotype between carriers of Northern or Eastern Europe or United States origin, who have higher percentage of small mutations, compared to Mediterranean and North African population, who very frequently carry deletions [14;15]. Different genetic-testing strategies can miss the diagnosis in the case of rarer pathogenic variants, such as atypical intronic variations, the presence of two *DMD* pathogenic variations, or even rarer uniparental disomy [16,17]. For optimum diagnostic yield in *DMD* gene analysis, the European quality assessment scheme (EMQN, *Eurogentest*) genetic guidelines advocate three levels of testing: first MLPA to identify deletions

or duplications, then full exon sequencing, to pick up small mutations, and thirdly RNA testing [18]. RNA-analysis requires RNA sourced from skeletal muscle tissue. Alternatively, since stem cells from a urine sample express dystrophin, they can be used as a surrogate source of RNA or protein [19].

AF then outlined use of the same three stage genetic test strategy proposed by the EMQN guidelines, for pre-conception carrier testing. This strategy has largely been adopted already within the EU and more widely, though with some country-based differences. Comprehensive genetic testing allows counselling at family-planning, pre-conception, pre-implantation, and prenatal stages. The advantage and added value of using tele-genetic counselling was described to facilitate admittance to high quality genetic expertise. Telegenetics seems acceptable to families because it allows wider and easier access for them to expert genetic advice without the need to travel long distances to centres of excellence (i.e.: those of the European Reference Networks (ERNs) [20]). In discussion about the most appropriate age for genetic testing of pre-symptomatic minors, it was accepted that this poses important ethical dilemmas and so, in practice, it is rarely undertaken except in clinically justifiable circumstances.

An important current 'unknown' is whether the dystrophinopathy phenotype can also be influenced by ageing. This because dystrophin expression may vary over time in different tissues or even in specific muscle types, as well as in the heart and brain. Dystrophin levels could theoretically increase through regeneration and 're-use' of stem cell patchiness in other tissues. If this occurs, it would be yet another variable influencing and confounding prediction of the severity of phenotype in *DMD*-carriers.

3.2 Chromosome interactions, X-chromosome inactivation and skewing

Joost Gribnau (JG) outlined what is currently know about sex-chromosome interactions and X-chromosome inactivation. In males, the way XY chromosomes interact is predictable but in females, XX-interactions are largely unpredictable. This is in part because – whereas an X-chromosome has 1,000 genes, the Y-chromosome currently has only 73 and through the course of evolution even this number has and continues to decrease further. The Y chromosome is not a suppressor of X-chromosome genes. The process of X-inactivation in females is necessary to prevent the lethal 'mixed messaging' when / if two Xs remain active. Inactivation is random but, in animal studies, follows a Gaussian distribution curve. Less is known about the effects of X-chromosome inactivation in humans because it occurs after day-14

post-conception, and current legislation doesn't allow researchers to continue human embryo studies beyond that stage of development. However, from knowledge of phenotype expression in about 180 X-chromosome linked conditions, it is known that many of those affected show a degree of mosaic tissue expression. JG explained, however, that as in mitochondrial disorders, the level of X-chromosome skewing in one tissue may be quite different from in another (e.g.: skewing in peripheral blood cells versus in skeletal or cardiac muscle). Technologies are currently exploring how to reactivate and inactive X-chromosome.

3.3 Skewed X-chromosome inactivation in prediction of DMD carrier phenotype?

Lidia Gonzalez Quereda (LGQ) summarised the literature on X-chromosome inactivation in DMD and other X-linked conditions and concluded that published reports are contradictory. This is partly explained by differences in testing methods of skewing and various definitions of its extent [21,22]. LGQ defined random skewing between X-chromosomes as less than 80:20, skewed as greater than 80:20 and > 90 as highly skewed. Most reports agree that extreme skewing predicts a worse phenotype in *DMD*-carriers. Rarely, some families have more often highly skewed X-chromosome patterns, probably explained by the inheritance of other co-factors [23]. Furthermore, the pattern of X-chromosome inactivation is a dynamic process in different tissues, potentially changing with age, over time and during the process of degeneration-regeneration [24]. It is theoretically possible that multiple nuclei can variably turn on and off within skeletal muscle and activate or deactivate in different patterns. In skeletal muscle, each regeneration cycle can result theoretically in a change in its X-chromosome activation / inactivation pattern. However, the need for more repair results in greater X-chromosome skewing as the number of stem cells reduces with ageing. This explains why determining the degree of X-chromosome skewing in blood cells cannot predict what is happening in other tissues or over time. It is estimated that 15% of cells expressing dystrophin are sufficient to prevent skeletal muscle from eccentric injury [25].

4.0 DMD female carriers: Overview of current management and treatment

4.1 Overview of care of males affected by DMD as a guide to management of *DMD* carriers

Anna Sarkozy (AS) discussed the extent to which management and therapies available for males with DMD can be applied to '*DMD*–carrier'. As in the case of males, the approach to testing and management of females should be related to their clinical stage. Optimum care starts with making a timely diagnosis as this is necessary before an affected individual can access the clinical assessments they need. Communicating a DMD diagnosis is devastating news to families and should take place with adequate time for discussion, answering immediate questions and setting out initial 'planning for the best'. The *standards of Care DMD* [26,27] define what is needed, when and by whom this should be provided as well as the frequency of assessments, nature of testing and schedules of follow-up appointments. For males with DMD, corticosteroid therapy is recommended from around four years of age onwards, to reduce the rate of functional decline. However, not all affected males respond to corticosteroid therapy and all steroids have a range of predictable side effects. Thus, careful monitoring is needed to arrive at the best risk-benefit balance, individualised to the patient / family. Daily steroid regimes are more effective than intermittent dosing in prolonging ambulation and preserving upper body strength, but daily regime also associates with more severe side effects, such as growth stunting, weight gain and osteopenia [28]. In the UK, a standardised ambulatory assessment (The *North Star Ambulatory Assessment)* has been developed and has been applied for over a decade to monitor motor abilities over time of DMD patients, and their response to therapies.

Although rare, males with somatic mosaicism for a pathogenic variant in the *DMD* gene have been reported to have a phenotype like that seen in *DMD*-carriers. They can present with an isolated cardiomyopathy, muscle asymmetry and / or a milder muscle phenotype [29,30].

On average, females at risk of being *DMD*-carriers are offered genetic testing at/above age 16 years and are recommended to be screened regularly for cardiomyopathy. However, it is unclear if any additional monitoring should be recommended after confirmation of carrier status in the absence of abnormal symptoms/signs, or baseline laboratory results. It is also unclear what symptoms/signs should prompt further investigations and/or require regular monitoring. Furthermore, there is no agreement on the best functional motor scale for *DMD*-carriers, given that a ceiling effect may be expected with the North Star Ambulatory assessment in carriers who are likely to be milder affected. Finally, it is unclear whether

corticosteroids should be offered to *DMD*-carriers who have skeletal muscle symptoms, and if so, at what age, which regime and how this should be monitored. There is also insufficient experience on minimum effective steroid dosing in adult females. Finally, further research is needed to define the prevalence, nature, and management of females with cognitive impairment attributable to their *DMD*-carrier status.

AS emphasized the importance of offering psychosocial support to *DMD*-carriers at the time of diagnosis. This would not only help females cope with their own genetic diagnosis but would also support them in the emotionally charged process of informing at-risk relatives of their genetic risk and need for testing.

4.2 Case series presentations from UK, Italy, and Netherlands

Aleks Pietrusz, Fernanda Fortunato and Saskia Houwen presented personal case series of *DMD*-carriers shown in Table 1 (Panel a, b, and c) that well highlight the complex clinical heterogeneity seen in *DMD*-carriers.

4.3 Outcome of EURO-NMD survey of female carriers

Teresinha Evangelista (TE) presented the results of two surveys of female carriers, developed as part of this workshop. The surveys sought responses from clinicians interested in neuromuscular diseases, patients, and relatives. Dissemination of the survey was via the ERN EURO-NMD site between May 2021 and January 2022.

Responses were combined from 94 respondents (66 females, 27 males and 1 undisclosed) in 12 European countries. The largest number of responses were from Greece (41), the UK (20) and Romania (14) and most respondents were patients. Other responders were in Belgium, France, Italy, Poland, Serbia, Spain, Sweden, Turkey, and Ukraine. For questions such as: "are you a *DMD* mutation carrier"; we have analysed the answers from the female participants separately. 79.1% of the responders had had their genetic status confirmed but 19.4% had not; one participant did not know whether she had been tested. Most females harboured out-of-frame duplications followed in prevalence by in-frame deletions. The mean age for testing was 21.4 years (range 7 months-54 years). About one third (31.3%) of females reported symptoms, mostly muscle weakness and pain, followed in prevalence by fatigue and cardiac involvement. Only 25.3% attended a specialised neuromuscular clinic, and for those, most attended either annually or alternate years. Some 53.7% regularly attended the cardiology clinic – mostly annually. The

majority (74.6%) had received genetic counselling. Three females were wheelchair users and one of these also needed NIV overnight. Very few clinicians responded (n=71) making it difficult to draw conclusions. Most responses were from neurologists and most of them had ten or less patients under follow-up. Most clinicians offer symptom review and cardiac assessment from time of diagnosis and all but two considered regular surveillance necessary for *DMD*-carriers. The interval between surveillance assessments varied from annually to every 3-5 years. Cardiac assessment and surveillance were considered extremely important and required life-long.

The number of responders to this first *DMD*-carrier survey was too small to allow definitive conclusions. Nonetheless, most respondents had had genetic test confirmation of their diagnosis. Thirty one percent reported symptoms, and few were followed in specialised clinics. Most clinicians, who responded, agreed that regular follow-up of carriers was appropriate, even if only for symptom review and cardiac assessment. It will be important to refine the questionnaires, broaden the scope of carrier-responders to capture those at the milder or asymptomatic end of the phenotype, and particularly to obtain responses from more cardiologists.

4.4 Ataluren treatment for DMD-carriers

Ataluren (*Translarna*) was developed following the discovery some 20 years ago that the aminoglycoside gentamycin allowed production of full-length dystrophin in mice who had non-sense dystrophin-mutations. About 13% of patients with DMD are suitable for this therapy and, in males, it may help to delay loss of ambulation and decline in respiratory muscle strength [31-33]. Luisa Politano (LP) presented encouraging data on the response to Ataluren therapy in one patient from a cohort of 355 *DMD*-carriers in Naples, Italy. Therapy was started because of early onset muscle weakness in that carrier and seemed to stabilize muscle function and allow return of ambulation following a leg fracture [34]. This experience is supported by results of an amalgamated series of carriers from different countries who had various severities of muscle weakness. Medication was well tolerated and either slightly improved ambulation or delayed loss of ambulation. It also appeared to stabilize respiratory and cardiac function over mean follow-up of 34 months [35]. Interestingly, treatment appeared to be more beneficial in

younger females with skeletal involvement. However, since the rate of deterioration is so variable in symptomatic *DMD*-carriers, these observations need to be validated by larger studies of longer duration.

5.0 From animal models to therapies for patients

5.1 Animal models of *DMD*-carriers – what can they tell us?

Inès Barthélémy (IB) discussed animal models for *DMD*-carriers, including some under evaluation, that might provide suitable mimics of the phenotypic range of human *DMD*-carriers. Among mouse models, mdx carriers exhibit no skeletal muscle nor cardiac involvement, unless unmasked by isoproterenol infusion [36]. Mdx/WT chimeras and Mdx/utrn$^{-/-}$/Xist$^{\Delta hs}$ mice present with skeletal muscle and cardiac involvement and can serve as small animal models of *DMD*-carriers [37,38]. Novel rat models under investigation might offer a complementary context to work on. The male pig DMD-model, with deletion of exon 52, has a severe phenotype but there are no clinical effects in female animals carrying this mutation. However, myocardial, and skeletal muscle histological anomalies are found in some individuals [39]. Golden retriever dogs with a splice-mutation in intron 6 (GRMD) have been studied also for their use as a model for human *DMD*-carriers. These dogs retain skeletal muscle function, but develop a form of cardiomyopathy, similar to that seen in carrier females. They manifest left ventricular dysfunction with fibrosis in the lateral wall and also develop ventricular arrhythmias [40]. This GRMD-carrier-model is currently the most promising one for study of therapies for cardiomyopathy in *DMD*-carriers.

5.2 Registries and Clinical Trials – research priorities and study designs

Michela Guglieri (MG) reviewed how creation of patients' registries allows collection of information about individuals affected by a specific condition [41]. Registries can have different purposes and range from epidemiological studies to providing ways of collecting longitudinal natural history data. Crucial to the success of a registry is that it has a well-defined purpose from its outset since that informs the type of data fields to be included. Currently, there are no established registries for *DMD*-carriers. However, data on *DMD*-carriers might be collected within existing registries for DMD, BMD, LGMD or general neuromuscular conditions; or in registries for specific clinical manifestations such as cardiomyopathy. Design of a *DMD*-carrier registry needs to take account of the fact that many carriers will be

asymptomatic, the variable presentation and diverse specialties to whom female carriers may present, and inconsistency in follow-up and the care provided to patients. There was consensus on the preferability to have a separate, specific registry for *DMD*-carriers, where data input from both patients and clinicians can occur. Depending on their purpose, registry could help to inform the different clinical presentations of *DMD*-carriers as well as support the development of clinical trials and drug development.

Trials in DMD-carriers

Several therapeutic approaches, targeting different aspects of muscle pathophysiology, have been developed and tested for males with DMD over recent years, and others are under study for patients with BMD [42,43]. Some, such as exon skipping and gene therapy using micro-dystrophins, would not be suitable for females affected by dystrophinopathies, but other molecules, targeting down-stream processes of dystrophin deficiency could be considered. However, it is premature to consider trials in *DMD*-carriers until there is a better understanding of the muscle pathology in carriers and how it progresses or changes over time. The lack of validated animal models of the human-carrier skeletal muscle phenotype to provide robust, reproducible pre-clinical data on which to plan human trials is currently also a barrier [44]. Females with dystrophinopathies can present with isolated skeletal muscle, cardiac, respiratory or brain involvement or a combination of these effects. Therefore, the specific target of the selected intervention or treatment for study would need to be pre-defined to guide clinical trial design, inclusion and exclusion criteria and outcome measures.

Learning from the experiences of human trials in males with DMD, a major barrier to the design of clinical trials in *DMD*-carriers is the lack of natural history data to allow definitions of study endpoints of muscle strength and function, cardiomyopathy and associated comorbidities and the factors that affect trajectories of each. Upcoming natural history studies in males with BMD may help in the design of studies in *DMD*-carriers.

In the face of these difficulties, a number of simultaneous approaches could be adopted to advance our knowledge about *DMD*-carriers: (1) retrospective studies to provide understanding about how symptoms and phenotype expression evolve in females with dystrophinopathies; (2) prospective natural history studies to describe the nature, prevalence and severity of symptoms and comorbidities and to determine

rates of progression and change; (3) patient registries to provide the full spectrum of clinical presentation in *DMD* carriers – bearing in mind that the majority are asymptomatic and so may be difficult to reach.

6.0 Extra-skeletal involvement, surveillance, and management

6.1 Outcome measures and muscle magnetic resonance imaging

Erik Niks (EN) reviewed the utility of muscle imaging in patient assessments. Imaging of muscles originally began using ultrasound and then progressed to using computer tomography (CT) - even before genetic testing was routinely available. However, standard and, more recently, quantitative muscle magnetic resonance imaging (MRI) is able to provide clinically useful correlations between imaging abnormalities, functional assessments (e.g.: NSAA, NSAD, PUL, FVC, etc) and muscle biopsy findings. EN reported results from a qualitative MRI study of skeletal muscle in 12 patients [45]. Qualitative assessment evidenced marked asymmetry of findings on T1-weighted images, including in some participants with no muscle symptoms or signs, while STIR-T2 images were normal. Muscle MRI also allows quantification of fat content within muscle, similar to the results from muscle biopsies. Imaging findings were similar in male patients with BMD or DMD. Longitudinal changes in muscle MRI-findings have also been studied extensively in DMD and correlated with changes in functional assessments. A 10% increase in fat-fraction in the lower limb denotes a 4-fold increase in risk for loss of ambulation. A similar increase in biceps fat-fraction equates with a 3-fold risk of losing hand-to-mouth feeding ability. Conversely, no significant changes in function were evident despite MRI changes in patients with BMD over one year follow-up. Interestingly, quantitative muscle-MRI has also confirmed a proximal to distal gradient in changes within the same muscle. Changes in MRI of the diaphragm may allow similar prediction in terms of loss of respiratory muscle strength, but this requires further longitudinal study to correlate MRI and FVC-measures [46-48].

EN concluded that muscle MRI could reduce current reliance on muscle biopsies and that MRI changes are evident before onset of symptoms and signs. He also drew attention to the fact that muscle findings were commonly asymmetric in symptomatic *DMD*-carriers – something that needs to be taken into account in functional assessments.

6.2 Cardiac surveillance and cardiomyopathy in *DMD*-carriers

John Bourke (JB) explained that whereas much is known about patterns of dystrophin expression in skeletal muscle from biopsy studies in *DMD*-carriers manifesting skeletal muscle symptoms, this information is almost completely lacking for the heart. The risk of complications inherent in obtaining heart tissue, means that it is rarely justified to perform a cardiac biopsy. However, unlike in skeletal muscle, there is no scope for myocardial regeneration because there are no cardiac stem cells to allow cell proliferation. However, from animal models with the mdx3cv mouse, it is known that as few as 4% of mosaic dystrophin expressing cells preserve LV-function, whereas uniform expression of 3.3% dystrophin in all cells does not [49]**.** Upregulation of utrophin expression also helps preserve heart function and happens in all cells.

So, in *DMD*-carriers it is the pattern in mosaic expression that is critical to whether a clinical cardiomyopathy develops and not just whether dystrophin is present at all or not [49]. In the human context, it has been estimated that having more than 50% of cells expressing dystrophin results in only mild LV-dysfunction or prevents cardiomyopathy altogether [49]. Theorising from these considerations, it follows that we do not know what the net effect of the interaction between endogenous and a synthetic, shorter dystrophin resulting from gene-therapy, in *DMD*-carriers would be.

JB then reviewed how concerns about cardiomyopathy in *DMD*-carriers came to light originally with publication of the 193-carrier cohort series from the Naples group in which 84% females had cardiac test abnormalities consistent with heart involvement [50]. Subsequent studies confirmed this finding [51-53] and a meta-analysis summarised the risk of cardiac dystrophinopathy in *DMD*-gene carriers as 7.3-16.7% and 0-13.3% for *BMD*-gene carriers [54]. The explanation for such wide ranges is prevalence relates to how cardiomyopathy is defined in different reports – by increased LV-chamber dimensions alone, presence of segmental dysfunction with preserved global function, reduced global-function as measured by diverse thresholds of left-ventricular ejection fraction (LVEF%) or fractional shortening (FS%).

JB then addressed the question of whether LV-dysfunction can develop *de novo* during surveillance, despite a previously normal baseline assessment from an analysis of the Newcastle cohort (unpublished data). Possible cardiomyopathy was defined as LVEF < 55% and/or FS < 28% and definite

cardiomyopathy by LVEF ≤ 40% and/or FS ≤ 25%. From a retrospective analysis of 53 *DMD* carriers (33 with mutations associated with *DMD*-gene, 20 with *BMD*-gene), who had serial assessments and at least nine-year (range 9-22) follow-up, the study confirmed that LV-dysfunction can develop despite a normal initial echo-assessment. This supports the view of other workshop participants that *DMD* carriers need continued cardiac surveillance, regardless of the findings at their baseline assessment. All these females received combination heart medications from the point of earliest detection of global or segmental LV-dysfunction [55]**.**

In discussion, JV reported that a third of *DMD*-carriers >50 years of age in his series from Denmark reported cardiac symptoms, and a third were taking regular cardiac medications (personal observation). He also pointed to the value of prominent R-wave voltages in the right precordial ECG-leads as a neglected sign of cardiac involvement in *DMD*-carriers, the increase in echo-test sensitivity by measuring global longitudinal strain and of stress echo-evaluation and the additional information obtainable from cardiac MRI (cMRI) in carrier surveillance.

JB then summarised the predominantly echo- and ECG-based carrier surveillance schedule operating in Newcastle formerly, and how cMRI is now being used increasingly to clarify echo-uncertainties, or to allow lengthening out of follow-up intervals from three to five years. The need for cardiac testing is typically triggered by the new diagnosis of a boy with DMD in the family, and ACE-inhibitor and mineralocorticoid antagonist is recommended on finding any echo-evidence of LV-dysfunction or confirmation of fibrosis on cMRI, if that test is performed. JB recommended a reduced threshold for cMRI testing in *DMD*-carriers, particularly if the results are likely to change management.

He also highlighted some problems with the current schedule, in that asymptomatic cardiac dystrophinopathy is sometimes already evident on first testing a *DMD*-carrier and many, with normal initial assessments, lapse from subsequent cardiac follow-up altogether. Better education of *DMD*-carriers routinely about the reasons why cardiac surveillance is recommended could help correct this.

6.3 Cardiac biomarkers: state of the art

Anca Florian (AF) highlighted the lack of longitudinal studies addressing the trajectory of decline of LV function over time in *DMD*-carriers who develop cardiac dystrophinopathy [53,56]. She reiterated the point about the lack of a uniform definition in the echocardiographic literature of when a *DMD*-carrier should be considered to have developed cardiac dystrophinopathy.

The current gold-standard to define heart involvement by echocardiography is LVEF% measured by the Simpson's biplane method, which sums up LV-systolic function in a single figure [54]. However, this is a measure of global LV-systolic function, and the method does not take account of posterior LV-wall function. This makes it insensitive in detecting early signs of segmental LV-dysfunction which typically first becomes evident in the postero-lateral / postero-basal LV-segments in cardiac dystrophinopathy [57]. This combination of confounders means that echocardiographic measures of LV-function are not yet suitable for use as primary endpoints for studies in females with cardiac dystrophinopathy.

Progressive cardiac fibrosis with a non-ischemic, "myocarditis-like" pattern is the hallmark of cardiac involvement in males with DMD or BMD and in *DMD*-carriers, and the same pattern is seen in all [58]. In conjunction with specific genotypes and plasma CK levels, cMRI allows earlier detection and evaluation of myocardial fibrosis as well as measures of LV-function [8].

Based on current available data, cardiac biomarkers, including troponins and natriuretic peptides seem unreliable for the early detection of heart involvement in *DMD*-carriers. Novel biomarkers from the MicroRNA class, like reduced micro-RNA 29C levels correlate with the presence of myocardial fibrosis on cMRI and so may prove useful in screening for when and in which carriers to perform cMRI [59-61].

Genetic status is not determined in potential *DMD*-carriers until at least age 18 years in most countries, unless there are other manifestations of the condition, and the course of cardiomyopathy in this sub-group may not be representative of what happens in the majority.

AF advocated greater clinical use of cMRI in the assessment of *DMD*-carriers to ensure earlier access to therapies for those are likely to benefit from them. However, the lack of robust cardiac natural history data in *DMD*-carriers, in particular on the rate of progression of early asymptomatic abnormalities, prevents firm recommendations about the optimum timing of an initial heart assessments by cMRI.

6.4 Thresholds and therapies for cardiac management

Linda Cripe (LC) outlined the many uncertainties and shortcomings in arrangements for cardiac surveillance of *DMD*-carriers with respect to their risk of cardiomyopathy. There was little agreement on the optimum age to initiate heart testing, for example. The more sensitive the heart assessments performed, the more abnormality will be detected and at younger ages. After sons with DMD have died, their carrier mothers tend to lapse from cardiac follow-up themselves, to the extent that we do not really understand what happens to these females in later life or even whether their life expectancy is affected by their abnormal genetic status.

LC suggested that much of what evidence is lacking could probably be obtained from registry data and routine enquiry of *DMD*-carriers about premature deaths of females in previous generations. There is also a lack of agreement in some quarters about whether cardiac screening should be undertaken at all, if we do not have evidence of the benefits of early initiation of conventional heart medications [51,62,63]. The availability of gene therapies could change the therapy paradigm, but it is not clear if *DMD*-carriers would benefit from such therapy.

Despite all these uncertainties, LC advocated regular testing of *DMD*-carriers for evidence of cardiac dysfunction at about 3-yearly intervals, and the role of cMRI testing when the echo-assessments are normal to increase the sensitivity of finding the earliest evidence of abnormalities. Finding any degree of LV-dysfunction clearly justifies stating ACE-inhibitor therapy and the presence of accompanying fibrosis justifies combining this with a mineralocorticoid antagonist (ie: spironolactone or eplerenone). It is less established whether evidence of myocardial fibrosis in the context of normal LV-function measures should trigger the same therapies. This is mainly because definitions of cardiomyopathy from other aetiologies do not readily apply to this 'pre-dysfunction' stage and the rate of progression from fibrosis to dysfunction in *DMD*-carriers has not been studied adequately. Intuitively however, because the presence of fibrosis confirms that the heart is already 'affected', it seems appropriate to discuss starting therapy with patients in that category too.

The availability of newer, more potent heart medications such as sacubitril-valsartan, sodium-glucose co-transporter-2 (SGLT2) inhibitors and novel anti-fibrosis agents increase the options for those whose heart function continues to decline despite earlier combinations of therapy and allow for more individualised

approaches in drug selection [64]. LC also raised several novel considerations, including whether the chronic stress of caring for affected son(s) contributes to the risk of developing cardiomyopathy in *DMD*-gene carrying mums (i.e.: Takosubo's LV-dysfunction mechanism), and whether these females have a higher prevalence of acquired heart disease in later life.

In conclusion, LC advocated for education of cardiologists regarding the importance of cardiomyopathy surveillance of *DMD*-carriers. A single normal echocardiogram was not in itself reassurance of long-term cardiovascular health. She stressed the need to provide more information and guidance both for clinicians and patients. Females who are at risk need to be better informed about the implications of carrying a *DMD* pathogenic variant and to be less neglectful of their own health in the face of carer responsibilities for their affected child/children. There also needs to be a more concerted effort to provide psychosocial support to females who carry the dystrophin mutation. Not only is there a significant burden to these women as caretakers but as at-risk individuals for significant cardiomyopathy.

7.0 General discussions & recommendations

We describe here below the key themes emerged during the discussion at the workshop and provide recommendations.

7.1 Definition of *DMD*-carriers.

There was general agreement that the term '*DMD* gene carrier' does not distinguish adequately between females who carry a pathogenic *DMD*-gene variant but have no symptoms or signs and those who present with clinical features. To increase access to appropriate care, the WS participants recommended limiting the use of 'carrier of *DMD*-gene pathogenic variant' or '*DMD*-carrier' to females without symptoms or signs and referring to those with abnormal findings as 'patients with dystrophinopathy'. It was also highlighted that there is need to avoid using the term "manifesting carrier", for this latter group of individuals as this may create confusion regarding their clinical status. The participants also considered that further sub-classification, according to the selective organ/tissue involved could be helpful clinically (i.e.: skeletal muscle dystrophinopathy; cardiac dystrophinopathy; cognitive dystrophinopathy, etc). It

was also agreed that the degree of X-inactivation level was unhelpful in defining the clinical status of affected females currently and it should not be part of the genetic diagnostic settings.

7.2 Need to define Minimum Care standards for *DMD*-carriers.

Accepting the many gaps in current knowledge and understanding and the different phenotypes with which a *DMD*- carrier may present, participants agreed to recommend the following minimum set of care standards on the basis of their expert consensus:

I. Genetic Testing:

a. Access to genetic counselling and confirmation of *DMD*-carrier status should be available routinely to all at-risk females from around age 16 years. However, *DMD*-gene testing should also be available at a younger age to females who are at risk of being carriers and who present with any symptoms or signs associated with DMD/BMD phenotype, including learning and cognitive difficulties.

b. X-chromosome inactivation analysis is not a validated diagnostic test.

c. The age of genetic testing is arbitrary but should also take into account the need for young females to have their carrier status and genotype clarified before contemplating pregnancy.

d. *DMD*-carriers are typically expected to take responsibility for communicating their diagnosis and the advisability of assessment to other relatives who may also be carriers. A leaflet could help make this task easier, and the WS recommends reviewing what patient-orientated literature is already available and developing an updated version if needed to be issued to confirmed *DMD*-carriers.

II. Neuromuscular assessment:

a. Minimum care standard for *DMD*-carriers should include referral to a neuromuscular specialist for a comprehensive assessment, including CK measurement(s). First review should take place within 6-12 months from time of genetic confirmation of *DMD*-carrier status.

b. In the case of normal first assessment, clinical review (with CK measurement) should be offered again in ~3-5 years. Further investigations, including respiratory assessment and muscle imaging can also be recommended by specialist(s), as appropriate.

c. Subjects with persistent muscle symptoms (i.e.: weakness, easy fatigue; pain, respiratory symptoms) should continue to have regular neuromuscular assessment by a specialist neuromuscular or

rehabilitation team. Assessment by neuromuscular specialists allows patients to access disease modifying therapies (e.g.: steroids; ataluren, etc) and opportunities to participate in trials and other evaluations of promising novel medications and therapies.

III. *Cardiac surveillance:*

a. Referral for detection and ongoing surveillance of cardiomyopathy should take place within six months of genetic confirmation of *DMD*-carrier status and, to ensure it happens consistently, referral for cardiology assessment should be an outcome of the initial neuromuscular team assessment.

b. Cardiac evaluations should include standard 12-lead ECG (+/- Holter ECG) and non-invasive imaging (transthoracic echocardiography or cMRI) of the heart to detect evidence of global or segmental LV dysfunction. Finding of cardiac fibrosis on cMRI, if performed, means that LV-dysfunction will probably develop later, even if systolic function is normal at that stage.

c. Because cardiomyopathy can develop *de novo* later in subjects with initially normal tests, carrier females should continue to follow a schedule of cardiac checks at 3–5-year intervals. That interval can depend on the sensitivity of imaging used in screening and the results of latest assessment (e.g.: echocardiography, 3 year or cMRI, 3-5-year intervals).

d. When testing confirms either segmental or global LV-dysfunction and even in the absence of any other symptoms or signs, heart medications should be started. This will usually consist of a combination of an ACE-inhibitor or angiotensin-receptor blocker and a mineralocorticoid receptor antagonist.

e. For carriers who develop essential hypertension, it is preferable to use an ACEi or ARB both to achieve desirable blood pressure lowering and mitigate the possibility of cardiomyopathy developing later.

7.3 Increase awareness

WS participants agreed on the need to increase awareness and understanding of *DMD*-carriers and particularly to:

I. Increase awareness for cardiac risks of *DMD*-carriers. Many females who carry a pathogenic variant in the *DMD* gene are unaware of the advisability of having heart assessments, and some cardiologists are unaware that these females are at risk of cardiomyopathy. Remedying these information deficits requires

development of a range of appropriate, readily accessible, educational materials in multiple formats and platforms. This would allow *DMD*-carriers and at-risk females, clinicians, and psychologists to access quality information. It could also support *DMD*-carriers and patients in asking for aspects of care, not being provided in a timely way or at all. Updated leaflets should be provided at time of genetic diagnosis of *DMD*-carrier and again a few years later at time of clinical reviews

II. Work with advocacy groups to increase awareness, with relevant information for *DMD*-carrier/at risk females, stressing the importance of taking care of their own health generally (e.g.: smoking cessation, blood pressure, diet and weight management, diabetes, lipid status, etc) and the importance of having various specific health-checks and follow ups.

III. Raise awareness and knowledge about *DMD*-gene carrier phenotypes and modes of presentation across the range of health care professionals in relevant specialties through presentations at scientific meetings and webinars/seminars.

7.4 Registries and research studies in DMD-carriers

DMD-carriers continue to be a neglected group both in clinical and research contexts, and there is considerable variation within and between countries in how services are provided and whether carriers have access to appropriate information, assessments, and the care they need. The WS acknowledged the lack of evidence from carrier-specific studies to inform best practice. Correcting these calls for a combination of initiatives, including development of dedicated registries, or allowing inclusion of *DMD*-carrier data in existing registries, to understand the natural history of the variable phenotypes. Academic research studies are needed to provide the evidence to guide the use of existing and novel therapies for separate aspects of the phenotype, such as cardiomyopathy or learning / cognition difficulties attributable to the pathogenic variants in the *DMD* gene. There is an urgent need to develop and validate appropriate study endpoints for use in 'carrier' research since, currently the lack of that information is a major obstacle to the design of clinical trials for these patients.

The WS participants agreed on the need for further collaborative research on key aspects related to *DMD*-carriers, including in particular: a) pathophysiology, b) pathology, c) role of X-chromosome inactivation,

d) cognitive / behavioural aspects e) role of corticosteroid therapy for female patients with dystrophinopathy and f) role of exercise.

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All participants declared no conflict of interest related to the workshop topic.

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Table 1. Case series of *DMD*-carriers are illustrated in Panel a, b, and c

Panel a. The Dutch Cohort of females which were symptomatic before the age of 16.

NL-cohort	**Total N = 12, mean follow-up duration 17.2 years**
Age, years *Median (range)*	23 (8-56)
Age at onset, years *Median (range)*	4 (0-13)
Age at diagnosis, years *Median (range)*	7,5 (0,8-30)
Deceased *N, yes (%)*	1 (8)
Age of death *Median (range)*	32 (-)
NIV *N, yes (%)*	1 (8)
Cardiomyopathy *N, yes (%)*	0
Bulbar Symptoms *N, yes (%)*	4 (33)
Neurocognitive (Autism +/- LD) *N, yes (%)*	3 (25)
Ambulant *N, yes (%)*	8 (67)
Age LoA, years *Median (range)*	20 (15-30)
Scoliosis *N, yes (%)*	2 (16)
Muscular weakness *N, yes (%)*	7 (58)
Steroid treatment *N, yes (%)*	1 (8)

NL: Netherlands; NIV: Non Invasive Ventilation; LD: Learning Disability; LoA: Loss of Ambulation

Panel b. Two cases of "incidental" *DMD* copy number variations (CNVs) identified by prenatal array-CGH in females without any history of dystrophinopathy.

Case	Age	Family history	Reason of referral for Array-CGH	Array-CGH result	MLPA analysis	CK [U/L]	Muscle symptoms	Mobility	Cardiomyopathy
1	33y	Negative	U/S fetal abnormality in the pregnancy: mild fetal cerebral ventriculomegaly (Array-CGH performed on parent-fetus trio)	Duplication of 319 Kb at Xp21.1 (partially involving *DMD* gene)	Duplication of exons DP427c-ex1 and Dp427m-ex1 of *DMD* gene	N/A	NO	Ambulant	N/A
2	36y	Negative	U/S fetal abnormality in the pregnancy: increased NT (Array-CGH performed on parent-fetus trio)	Deletion of 164 Kb at Xp21.1 (partially involving *DMD* gene)	Deletion of exon DP427c-ex1 of *DMD* gene	N/A	NO	Ambulant	N/A

MLPA analysis was performed for confirmation of array-CGH results and clinical data of two females carriers collected.

U/S: ultrasound; CK: creatine kinase; N/A: not available; NT: nucal translucency

Panel c. Clinical findings, gene mutation and creatine kinase levels in manifesting carriers of *DMD* gene in London, Queen Square cohort.

ID	Age	Gene mutation	Age at onset of symptoms onset	CK [U/L]	Corticosteroids	Mobility	Cardiomyopathy
UK-1	22y 8m	Deletion exons 42-43	Approx. 0-2y	1570	Yes	Ambulant	No
UK-2	**26y**	Xp21 translocation	Approx. 2y	336	Yes	Non-ambulant	No
UK-3	36y 8m	Deletion exons 7-34	9y	N/A	Yes	Ambulant	No
UK-4	52y 7m	Deletion exons 22-29	21y	699	Yes	Non-ambulant	Yes
UK-5	22y 2m	Duplication exons 2-42	<5y	N/A	Yes	Non- ambulant	Yes
UK-6	18y 4m	Deletion exons 45-50	3y	6060	No	Ambulant	No
UK-7	22y 7m	Duplication exons 12	2.5y	1600	No	Ambulant	Yes
UK-8	37y 8m	Deletion exons 6-44	34y	1861	No	Ambulant	No
UK-9	40y 4m	Deletion exons 45-50	<5y	1032	No	Ambulant	Yes
UK-10	43y 2m	Mutation exons 34	Approx. 0-2y	237	No	Non-ambulant	Yes
UK-11	51y 1m	Unknown (diagnosed following the familial genetic screening – results N/A)	49y	779	No	Ambulant	No
UK-12	52y 8m	Unknown (diagnosed following the familial genetic screening – results N/A)	26y	1028	No	Ambulant	Yes
UK-13	56y 6m	Mutation c.433C>T (p.Arg145X)	54y	738	No	Ambulant	Yes

All patients (N=13) presented with various degree of lower limb muscle weakness and 12 (92.3%) with upper limb muscular weakness. Moreover, 23% (N=3) had bulbar function symptoms (all ambulant). Furthermore, 23% (N=3) required Non-Invasive ventilation (NIV), one before and two after loss of ambulation.

Five patients were on corticosteroid therapy. Two stopped due to side effects, three continued (including one experiencing side effects). Side effects included: weight gain, mood swings, bilateral cataracts. Patient highlighted in black passed away.

CK = creatine kinase; N/A: not available.