Targeting the secondary defects in Duchenne muscular dystrophy

Chapter 2

2 Other therapeutic approaches

Since at present there is no cure for DMD, there are other, mainly pharmacological, approaches that attempt to combat the symptoms caused by the underlying genetic defect. In this chapter an overview of the different players, and thereby targets, in the DMD pathology is given, together with compounds/approaches that act on those targets and have been used/tested in DMD patients or dystrophic animal models. A schematic overview of the pathways involved in the pathology and the main approaches acting on these parts is given in fig. 2.1. Table 2.1 gives an overview of all compounds, except from those discussed in chapter 1, currently in clinical trials for DMD and/or BMD. The overview in this chapter is certainly not an exhaustive list of all compounds ever tested in context of DMD or related diseases, which might relevant for DMD too, but aims to give a broad overview of possible approaches and their current status/results. In particular it aims to provide insight in which groups of compounds, with most promising outcome or mechanism of action, could be tested to try to enhance the therapeutic effect of AONs.

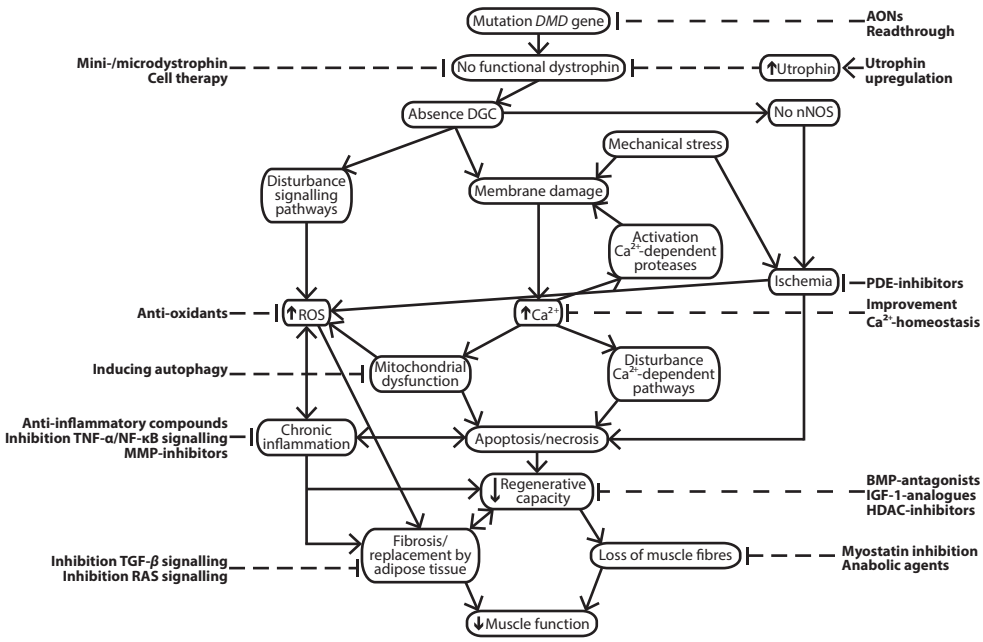

Fig. 2.1: Schematic overview of DMD pathology and therapeutic strategies

In DMD the primary defect, the absence of a functional dystrophin protein due to reading frame disruption or stop codon introducing mutations in the *DMD* gene, causes a cascade of downstream effects, including disturbance of calcium homeostasis, chronic inflammation and increase of reactive oxygen species (ROS). This in turn eventually leads to a loss of muscle fibres, which are replaced by fibrotic and adipose tissue, resulting in a loss of muscle function.

The therapeutic approaches described in this thesis and where they address the pathology are indicated.

Trial registration[a]	*Compound*	*Paragraph*[b]	*Primary mechanism*	*Phase*	*Primary Purpose*	*Patients*[c]	*Status*[d]
NCT01603407	Prednisone or deflazacort	2.2	Anti-inflammatory	III double-blinded	Safety/efficacy (optimal regimen)	Age 4-7 years; ambulant	Ongoing
NCT01580501	Tadalafil and sildenafil	2.3	PDE5-inhibitors	I open-label	Safety/efficacy	Age 7-15 years; ambulant	Ongoing
NCT01359670	Tadalafil or sildenafil	2.3	PDE5-inhibitors	? open-label	Safety/efficacy	Age 7-15 years; ambulant	Completed
NCT01350154	Sildenafil	2.3	PDE5-inhibitor	IV double-blinded, placebo-controlled	Efficacy	BMD Age 18-80 years; reduced cardiac function	Completed
NCT01168908	Sildenafil	2.3	PDE5-inhibitor	II double-blinded, placebo-controlled	Efficacy	DMD/BMD Age 18-50 years; reduced cardiac function	Suspended[e]
NCT01865084	Tadalafil	2.3	PDE5-inhibitor	III double-blinded, placebo-controlled	Efficacy	Age 7-14 years; ambulant	Ongoing
NCT01070511	Tadalafil	2.3	PDE5-inhibitor	IV double-blinded, placebo-controlled	Efficacy	BMD Age 18-55 years	Completed
NCT00758225	Idebenone	2.4	Anti-oxidant	II open-label extension	Safety/efficacy	Previous studies[423,424]	Completed
NCT01027884	Idebenone	2.4	Anti-oxidant	III double-blinded, placebo-controlled	Safety/efficacy	Age 10-18 years	Ongoing
NCT01126697	Coenzyme Q10 and/or lisinopril	2.4/2.8	Anti-oxidant/ACE-inhibitor	II/III open-label	Safety/efficacy	DMD/BMD/LGMD Age>8 years; preserved cardiac function	Ongoing
NCT01183767	Epigallocatechin gallate	2.5	Anti-oxidant/blocking IKK activity	II/III double-blinded, placebo-controlled	Safety/efficacy	Age 5-10 years; ambulant	Ongoing
NCT01856868	(-)-Epicatechin	2.5	Anti-oxidant	I/II open-label	Safety/efficacy	BMD Age 18-60 years; ambulant	Ongoing
NCT01335295	Flavocoxid	2.5	Anti-inflammatory/anti-oxidant/ NF-kB inhibition	I open-label	Safety	Age 4-16 years; ambulant	Unknown[f]
NCT01388764	L-arginine	2.6	Anti-inflammatory/anti-oxidant	I open-label	Safety/efficacy	Age 7-11 years; ambulant	Completed
NCT01099761	ACE-031	2.7.1	Myostatin inhibition	II double-blinded, placebo-controlled	Safety/efficacy	Age>4 years; ambulant	Terminated[e]
NCT01239758	ACE-031	2.7.1	Myostatin inhibition	II open-label extension	Safety/efficacy	Previous study (NCT01099761)	Terminated[e]

Trial registration[a]	*Compound*	*Paragraph*[b]	*Primary mechanism*	*Phase*	*Primary Purpose*	*Patients*[c]	*Status*[d]
NCT01519349	Follistatin344 (AAV-mediated)	2.7.1	Myostatin inhibition	I open-label	Safety	BMD/SIBM Age>18 years	Ongoing
NCT01847573	HT-100 (halofuginone)	2.7.2	Inhibiting TGF-β signalling	I/II open-label	Safety	Age 6-20 years	Ongoing
NCT01978366	HT-100 (halofuginone)	2.7.2	Inhibiting TGF-β signalling	II open-label extension	Safety	Previous study (NCT01847573)	Ongoing
NCT01982695	Lisinopril or losartan	2.8	ACE-inhibitor or AT1-antagonist	? double-blinded	Efficacy	Impaired cardiac function (ejection fraction <55%)	Completed
NCT01207908	IGF-1	2.9	Increase IGF-1	I/II single-blinded (vs corticosteroids)	Safety/efficacy	Age>5 years; ambulant	Ongoing
NCT01761292	Givinostat	2.11	HDAC-inhibitor	I/II open-label	Safety/efficacy	Age 7-11 years; ambulant	Ongoing
NCT01521546	Eplerenone	2.15	Aldosterone antagonist	? double-blinded, placebo-controlled	Efficacy	Age>7 years; preserved cardiac function	Ongoing
NCT01648634	Nebivolol	2.15	β-blocker	III double-blinded, placebo-controlled	Efficacy	Age 10-15 years; preserved cardiac function	Ongoing
NCT00606775	Carvedilol	2.15	β-blocker	IV open-label	Safety/efficacy	Age 8-45 years; preserved to moderate cardiac function	Unknown[f]
NCT00819845	Ramipril or carvedilol	2.15	ACE-inhibitor or β-blocker	IV open-label	Efficacy	DMD/BMD Age 2-45 years; preserved cardiac function	Unknown[f]
NCT01540604	CRD007	-	Anti-inflammatory (vascular)	II open-label	Safety/efficacy	DMD/BMD Age 2-11 years	Completed
NCT01826422	Docosahexaenoic fatty acid (DHA) and eicosapentaenoic fatty acid (EPA)	-	Dietary supplements (anti-inflammatory and beneficial effect on metabolic disorders)	II double-blinded, placebo-controlled	Efficacy	DMD/BMD Age 6-18 years	Ongoing
NCT01995032	L-citrulline and metformin	-	Dietary supplement and insulin sensitizer	III double-blinded, placebo-controlled	Safety/efficacy	Age 7-10 years; ambulant	Ongoing

Table 2.1: Overview clinical trials, currently ongoing and unpublished, of all compounds targeting secondary defects in DMD

[a]Registration on www.clinicaltrials.gov

[b]Discussed in chapter in this thesis

[c]DMD patients unless otherwise stated; BMD=Becker muscular dystrophy; DMD=Duchenne muscular dystrophy; LGMD=Limb girdle muscular dystrophy; SIBM= Sporadic inclusion body myositis

[d]Status on 1st of December 2013

[e]Based on preliminary safety data

[f]Status has not been verified in more than two years

2.1 Utrophin upregulation

Instead of trying to bring back dystrophin into muscle, an alternative strategy is to increase the expression of the dystrophin homologue utrophin. In the *mdx* mouse model it has been shown that transgenic overexpression of truncated utrophin ameliorates the dystrophic phenotype.[346] Full-length utrophin is even more efficient and could prevent pathology in *mdx* mice and improve the phenotype in CXMD dogs.[347-349] This started off high-throughput screens for drugs that can increase utrophin expression. Expression of utrophin is controlled by two isoforms of the utrophin promoter. The utrophin A is active in skeletal muscle and the utrophin B promoter in heart, lungs and endothelial cells. Most research focuses on the utrophin A isoform.[350] A cell model stably expressing the utrophin A promoter linked to luciferase has been used to assess the potential of thousands of drugs. One of these drugs, SMT C1100 was one of the most efficient hits. Its effect has been validated in the *mdx* mouse model, where oral treatment with this compound increased utrophin expression up to twofold, resulting into improved muscle histology and function. Unfortunately, a phase I clinical trial in healthy volunteers revealed that the drug plasma levels needed to induce an effect could not be obtained in humans.[351] Current research focuses on reformulation of this compound. A new trial in healthy volunteers with SMT C1100 in a nanoparticle aqueous suspension formulation resulted in higher plasma levels that were sufficient to increase utrophin levels in animal models. There are plans for a dose-finding phase Ib study in DMD patients.[352] A concern with utrophin upregulation approaches is that utrophin, in contrast to dystrophin, turned out not to be able to bind nNOS and restore its expression at the sarcolemma, whereas disturbances in nNOS function are important in DMD pathology.[353]

Biglycan is an endogenous protein that is present during development outside skeletal muscle fibres and cardiac muscle. It plays an important role in the regulation of signalling pathways and structural proteins, among which proteins (*e.g.* sarcoglycans, dystrobrevins, syntrophins and nNOS) that are part of the DGC.[354] Biglycan injection (local and systemic) in *mdx* mice resulted in upregulation of utrophin, recruitment of nNOS and improved muscle function and resistance to exercise-induced damage.[355] A possibility why biglycan, in contrast to utrophin alone,[353] could restore nNOS expression at the sarcolemma, is that biglycan also restores other compounds of the DGC, creating a more complete utrophin-associated complex, including nNOS.

The expression levels of utrophin in skeletal muscle are also controlled by post-transcriptional mechanisms, *e.g.* the activation of p38, which enhances the stability of utrophin mRNA. An activator of p38, heparin, was able to increase utrophin A expression in the diaphragm of *mdx* mice (ten days treatment with 500 UI/kg).[356]

2.2 Corticosteroids and other anti-inflammatory compounds

In healthy persons inflammation and activation of immune cells are beneficial for removing necrotic or damaged tissue after minor muscle damage and actually enhance muscle repair. However in DMD patients muscle damage is continuous.[357] Therefore, chronic inflammation is a prominent feature in DMD pathology. It was first thought to be mainly a non-specific secondary feature: the continuous damage to muscle fibres in the absence of dystrophin, the increased $Ca2^{+}$-influx and production of reactive oxygen species (ROS) were thought to lead to immune cells infiltration and the production of pro-inflammatory cytokines and chemok-

ines. However, it has become more and more clear that inflammation also plays an important specific role in the onset and progression of the disease pathology. Many different types of inflammatory cells (*e.g.* lymphocytes, macrophages, neutrophils, eosinophils and mast cells) have shown to play a role in the increased inflammation in DMD and *mdx* muscles. Large infiltrates of immune cells (mainly macrophages and $CD4^+$ T-cells) in muscle fibres are already observed before onset and in early stages of the disease. The characterization of this population is also distinct from that of non-specific inflammation following muscle injury, indicating an auto-immune component in the disease pathology. Furthermore muscle fibres from DMD patients have a large increase in major histocompatibility complex (MHC) I expression, which is normally lowly expressed by skeletal muscle cells, suggesting they can serve as potential antigen presenting cells.[357,358]

Both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T-lymphocytes levels are elevated in DMD. This T-cell activation is probably muscle-specific, since the frequency of activated T-cells was not elevated in *mdx* lymph nodes. Their role in aggravating pathology is shown by *in vivo* antibody-mediated depletion of T-cells, which reduced muscle pathology by around 60% ($CD4^+$ cells) to 75% ($CD8^+$ cells) in *mdx* mice.[359] In early stages $CD4^+$ T helper cells, which can activate other immune cells, are present. $CD4^+$ T-cells consist of two subpopulations: T_H1 $CD4^+$ T-cells and T_H2 $CD4^+$ T-cells. T_H1 $CD4^+$ T-cells produce IFN-γ and IL-12, which promote inflammation, but may have an attenuating effect on fibrosis. T_H2 $CD4^+$ T-cells are associated with IL-4, IL-5 and IL-13 cytokines and are strongly linked to fibrogenesis.[360] In addition, T-lymphocytes are an important source of TGF-β_1 (see paragraph 2.7).

Macrophages are also a large source of TGF-β_1 and platelet-derived growth factor (PDGF), a fibrogenic cytokine.[361] Depletion of macrophages in neonatal *mdx* mice resulted in a large decrease in muscle necrosis, suggesting they play a role in the early stages of the disease.[362] Macrophages can have phenotypically and functionally distinct subtypes. Classically activated M1 macrophages are activated by T_H1 cytokines IFN-γ and IL-12, express high levels of inducible NOS (iNOS) and promote tissue inflammation. Those are found first after acute muscle injury, cleaning up necrotic tissue and presenting antigens. Alternatively-activated M2 macrophages are activated by T_H2 cytokines IL-4 and IL-13, express high levels of arginase and suppress the T_H1 response.[363] Different M2 subtypes exist, of which M2c macrophages are seen in early phases and de-activate M1 macrophages. M2b macrophages have been observed in regenerating muscle after injury and M2a macrophages are abundant in final phases of tissue repair.[357]

Eosinophils are increased in DMD biopsies and can secrete IL-4 and IL-10, which promote T_H2 $CD4^+$ T-cell responses and thereby fibrogenesis. They also produce MBP-1, which attenuates the cellular immune response and promotes fibrosis. Knockout of MBP-1 in *mdx* mice (*mdx*/*Mbp-1*$^{-/-}$) results in a reduction in diaphragm fibrosis, without changes in macrophage content or inflammatory cytokines as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[364]

In addition mast cells and dendritic cells have been suggested to play a role in the pathophysiology of DMD, since the mast cell content is elevated in dystrophic humans, dogs and mice and activated dendritic cell infiltration is increased in dystrophic myofibres.[365,366] Furthermore *mdx* myofibres have been shown to be vulnerable to purified mast cell granule-induced necrosis.[367]

Considering the significance of inflammation in DMD pathology, several therapeutic strategies are based upon immune interventions.

Since at the moment no cure or therapy targeting the underlying genetic effect is available, most patients are treated with corticosteroids, most used are prednisone, prednisolone (prednisone is converted in the liver to its active form prednisolone) and deflazacort (an oxazolidine derivative of prednisone). Corticosteroids are 21 carbon steroid hormones, which have a wide range of actions, *e.g.* on carbohydrate and protein metabolism, lipid metabolism, electrolyte and water balance, the cardiovascular system, the neuromuscular system, lymphoid tissue and immune response.[368] Their exact mechanism of action in DMD is not known, but they probably reduce muscle necrosis and inflammation.[10,13,369] They might work partly via increasing $\alpha_7\beta_1$-integrin and laminin-2, thereby stabilising the DGC.[370] Prednisolone had a positive effect on muscle strength and histology (decrease in centrally located nuclei) in *mdx* mice.[371] Furthermore, in *mdx* mice it could reverse alterations in the brain. *Mdx* mouse brains show increased vascular permeability (impaired blood-brain barrier functionality) and reduced levels of several DGC-associated proteins and the Dp71isoform. These levels and the function of the blood-brain barrier were restored by prednisolone treatment.[372] In a dystrophin- and MyoD-deficient *C. elegans* model a reduction in the number of degenerating fibres was seen after prednisolone treatment.[369] As *C. elegans* only has a simple immune system, other mechanisms seem to be involved as well. One study in *mdx* mice suggests that deflazacort increases the calcineurin/NF-AT pathway. JNK1 activation in dystrophic muscle leads to an increased interaction and thereby nuclear restriction of the nuclear transcription factors of activated T-cells (NF-AT), which are regulatory proteins for hypertrophic growth of both cardiac and skeletal muscle. Deflazacort treatment leads to an increase of NF-AT target genes, among which the dystrophin homologue utrophin, thereby it decreases the dystrophic muscle fibre pathology.[373,374] An alternative possibility is that the anabolic effect of corticosteroids increases muscle regeneration and growth by enhancing proliferation of myogenic precursor stem cells or myoblasts.[10] Furthermore corticosteroids have a positive effect on Ca^{2+}-homeostasis, which is deregulated in DMD patients (see paragraph 2.12).[368] Unfortunately, corticosteroids also have deleterious side effects like osteoporosis, weight gain, growth inhibition, delayed puberty, mood swings and cataracts.[13,375-377] In unaffected individuals they have a catabolic effect on muscle, but in DMD this is generally abrogated by the positive effects.[378] Whilst in *mdx* mice prednisolone treatment is initially beneficial, it induces fibrosis and is detrimental to muscle function on the long term.[379,380] Notably, prednisolone induced fibrosis in the heart in mice, but this is not observed in DMD patients, where corticosteroid use prevents/delays ventricular dysfunction.[11,381-383] A comparative trial suggests that prednisone and deflazacort are equally effective at slowing disease progression, but that deflazacort causes less side effects than prednisone (less weight gain).[384] On average corticosteroids have shown to prolong ambulation for around three years, but, since they do not target the underlying cause, they cannot prevent the eventual loss of muscle fibres and function.[10] Therefore early start of treatment might be appropriate, since it cannot recover lost function.[385] In addition, not all patients tolerate long term use of corticosteroids, due to the numerous side effects. Several trials with prednisone have been conducted with altering treatment regimens, *e.g.* alternate day, weekend only or continuous low dose, to try to keep the positive effects, but reduce the side effects. However mostly less functional benefit was observed compared to the most widely recommended daily dose of 0.75 mg/kg/day or no reduction in side effects.[386-390] As there are many different dosing regimens for both prednisone and deflazacort, recently a large trial has been initiated comparing 0.75 mg/kg prednisolone daily and on an intermittent (ten days on, ten days off) dosing regimen and 0.9 mg/kg/day deflazacort (the dose equivalent to 0.75 mg/kg/day prednisolone) in

order to determine the most optimal balance between increasing efficacy and decreasing side effects and toxicity (FOR-DMD study, NCT01603407).[7]

Due to the side effects of corticosteroids after prolonged treatment, research has been conducted to find compounds with similar therapeutic actions, but fewer side effects. Serra *et al.* compared the efficiency of several non-steroidal anti-inflammatory drugs (NSAIDs) to methylprednisolone in *mdx* mice: two non-specific COX-inhibitors (aspirin and ibuprofen) and a selective COX-2-inhibitor (parecoxib). The anti-inflammatory effects of all drugs were largely comparable and resulted in amelioration of muscle morphology, with aspirin being slightly less effective and parecoxib slightly more.[391] In contrast, in another study, another specific COX-2-inhibitor (meloxicam) had little or no effect on exercise-induced muscle pathology in *mdx* mice.[392]

Another compound, HCT 1026, a NO-donating derivative of the NSAID flurbiprofen, was more potent than prednisolone in preventing muscle damage after one year treatment in *mdx* mice. Importantly HCT 1026 does not induce the side effects of corticosteroids.[393] It normalizes blood flow and thereby alleviated functional muscle ischemia.[394]

Doxycyline is an antibiotic member of the tetracycline family that, next to antimicrobial properties, also inhibits matrix metalloproteinases (MMPs; see paragraph 2.13) and reduces inflammation. Administration to *mdx* mice resulted in less inflammation, thereby protecting against the onset of myonecrosis on short term and reduction of pathology in both skeletal and cardiac muscle and improved muscle function in aged mice in the long run (nine months).[395] However, it must be noted that the dosage used was far higher than the human equivalent dosage used for its antimicrobial properties. Therefore additional studies are needed to test whether this dosage is applicable in humans.

Resveratol is a compound that can be found in food like grapes and red wine and has shown to have anti-inflammatory and oxidative metabolic enhancing properties. It acts through the Sirt1-PGC-1α pathway. A study treating nine weeks old *mdx* mice for eight months showed, via Sirt1 activation, a reduction of oxidative stress, loss of muscle mass and fibrotic tissue, but no reduction of inflammation.[396] The same treatment also resulted in amelioration of cardiomyopathy by Sirt1-mediated downregulation of p300, a pro-hypertrophic co-activator which is critical for cardiac development, but induces cardiomyocyte hypertrophy after overexpression.[397] Eight weeks treatment of younger (four weeks old) mice improved specific muscle force and short term (ten days) treatment of five weeks old *mdx* mice resulted in a decreased expression of inflammatory genes.[398,399] Utrophin is a downstream target of PGC-1α, but no increase in utrophin protein expression was observed in either study, although the last study did see a positive effect on utrophin mRNA levels.

Oxatomide (Tinset®), a histamine H1 antagonist used for the treatment of allergies, has anti-inflammatory effects by inhibiting the release of pro-inflammatory mediators from among others mast cells.[400] Furthermore, oxatomide has suppressive effects on antigen-presenting cells such as macrophages and dendritic cells.[365] In young, exercised *mdx* mice oxatomide improved whole body strength.[401] However in a pilot open-label trial in steroid-naïve DMD patients no improvement in muscle strength or function was observed after three or six months of treatment.[402]

2.3 NO-cGMP signalling pathway

NO is synthesized by nNOS and is an important regulator of contractile function and muscle

integrity in both skeletal and cardiac muscle.[403,404] In DMD and many other neuromuscular diseases the NO-cGMP signalling pathways are disturbed. Contracting muscle normally produces NO to ensure vasodilation and increased blood flow to fulfil the increased need of the working muscle for oxygen and nutrients. Lacking nNOS, contraction leads to a decrease in blood supply during contraction, sometimes resulting in micro-infarctions and thus increasing the amount of muscle damage. Hence a therapeutic strategy is to amplify NO-cGMP signalling by phosphodiesterase (PDE) inhibitors, mainly PDE5. These compounds are well known for their vasodilative properties in among others treatment of erectile dysfunction and heart failure.[405,406] A large drug screening in the dystrophin-deficient zebra fish *Sapje* identified the non-selective PDE-inhibitor aminophylline as a potential candidate to restore normal muscle histology. Aminophylline is known for its anti-inflammatory effects by inhibiting several inflammatory mediators. Thereafter also the PDE4-inhibitor rolipram and especially the PDE5-inhibitor sildenafil citrate (Viagra®/Revatio®) showed a positive effect.[407] Sildenafil citrate also improved respiratory and cardiac function in *mdx* mice.[408,409] The exact mechanism of the cytoprotective effects of sildenafil citrate is not known. Mitochondria are dysfunctional in *mdx* mice and DMD patients, resulting in reduced ATP production, thereby not meeting the energy demand of the muscle cells. Since the NO-cGMP pathway plays a regulatory role in mitochondria, it was thought sildenafil citrate-mediated PDE5 inhibition might have its beneficial effects by improving mitochondrial function. However, detailed analysis showed no effect of sildenafil citrate on mitochondrial function in *mdx* mice.[410] It probably exerts its effects by inhibiting Ca^{2+}-entry via transient receptor potential canonical channels (mainly TRPC6) and pro-inflammatory cytokines, like NF-κB and MAPKs.[411,412] An alternative hypothesis is that it has its positive effects by improving the blood supply to the muscles. Other PDE5-inhibitors were shown to be effective as well. The PDE5A-inhibitor tadalafil resulted in amelioration of muscle damage in *mdx* mice.[413] A subsequent clinical trial in BMD patients showed alleviation of muscle ischemia.[414] Several clinical trials with both compounds are currently ongoing: trials to determine if tadalafil and/or sildenafil citrate can improve muscle blood flow during exercise in DMD or BMD patients (NCT01359670, NCT01580501 and NCT01070511) and the effect of Revatio® (sildenafil citrate) on cardiac, muscular and cognitive function in BMD (NCT01350154). However a six months trial in DMD/BMD patients has recently been suspended (NCT01168908), due to negative results during an interim analysis. No benefit of Revatio was observed and, although group sizes were small, more subjects in the Revatio-treated group showed a worsening of cardiac function than in the control group, indicating it may even have adverse effects on cardiac function [presented at the Annual Connect Conference of the Parent Project Muscular Dystrophy, Baltimore, MA, USA, June 2013]. Tadalafil acts more specific, since it only stimulates cGMP, while sildenafil stimulates cGMP but also cAMP, which can result in putting too much stress on the heart. Furthermore, tadalafil has a better pharmacokinetical profile (longer acting) and a good safety profile. Pilot studies in DMD, mainly single dose-escalation studies, did not show serious adverse events and showed indications of protective effects to exercise induced damage. Therefore, a large phase III randomized, double-blind, placebo-controlled study in 306 DMD boys will start soon, examining the efficacy of 48 weeks of tadalafil treatment (NCT01865084).

Isosorbide dinitrate and MyoNovin (guaifenesin dinitrate) are NO-donating drugs. Short term treatment (18 days) of *mdx* mice had a positive effect on the stem cell population and resulted in accelerated fibre regeneration, especially in combination with prednisolone treatment.[415] A clinical trial in healthy volunteers to compare the pharmacokinetics of isosorbide

dinitrate and the NSAID ibuprofen and mainly to define possible drug interactions of a combined administration of both compounds is currently ongoing, in preparation for a trial in DMD patients (NCT01478022).

2.4 Anti-oxidants

As mentioned above oxidative stress plays a role in DMD pathology. One of the pathways mediating this response is via nNOS. nNOS produces NO, an important modulator involved in the regulation of numerous processes, *e.g.* muscle contractility, glucose uptake and repair. Normally nNOS interacts with the DGC, but it is dislocated in DMD (see paragraph 1.2.2). In itself the absence of nNOS does not lead to muscular dystrophy, but in dystrophin-deficient muscles it may aggravate the damage via, among others, increasing NAD(P)H oxidase activity and increasing inflammation and decreased blood flow to muscle due to impaired vasodilation. However oxidative stress itself probably also contributes to the dislocation of nNOS and the disruption of the DGC, by activating the NF-κB pathway and caveolin-3 signalling pathway.[416]

In *mdx* mice pathology differs between limb muscles and the diaphragm. Limb muscles undergo various cycles of necrosis, inflammation and degeneration around the age of two to eight weeks and show massive regeneration, which compensates for the loss of necrotic fibres, from three to 12 months, after which it starts to decline.[417,418] In contrast the diaphragm displays continuous and progressive degeneration throughout the life-span, combined with ineffective regeneration leading to muscle fibre loss, more resembling the human DMD pathology.[419] This difference in degeneration/regeneration in combination with the increase oxidant production observed in the *mdx* diaphragm compared to skeletal muscle, makes the diaphragm extra vulnerable for oxidative stress.[416]

Considering its role in DMD pathology, several strategies aim to target the oxidative stress. One class of compounds targets the NF-κB pathway (see paragraph 2.5). Furthermore several other anti-oxidants have been tested at various levels in DMD.

Coenzyme Q10 is essential for several enzymatic steps in the production of energy and functions as an anti-oxidant. In a pilot clinical trial addition of coenzyme Q10 to prednisolone treatment improved muscle strength.[420] A clinical trial with coenzyme Q10 alone or in combination with the ACE-inhibitor lisinopril is currently ongoing (NCT01126697; see paragraph 2.8). Another clinical trial comparing coenzyme Q10, prednisone or a combination in non-ambulant patients has been terminated, since the American Academy of Neurology now recommends that all patients with DMD should be offered treatment with corticosteroids.

Idebenone (2-(10-hydroxy-decyl)-5,6-dimethoxy-3-methyl-[1,4] benzoquinone; Catena®), a synthetic analogue of coenzyme Q10, is a compound that reduces oxidative stress by acting as a free radical scavenger and it improves mitochondrial function by increasing ATP-production and protecting the mitochondria against lipid peroxidation.[421] In the *mdx* mouse model idebenone treatment resulted in a reduction of inflammation and lower levels of fibrosis in heart, thereby normalizing heart function and improving voluntary running.[422] In a small phase I/II clinical trial in DMD patients no significant difference in heart function was observed. However, this study was not ideal due to the small number of patients and the fact that the idebenone-treated group was significantly older than the placebo-treated group. Nevertheless, the forced vital capacity of treated patients was better than those of

control patients, suggesting a protective effect on respiratory function.[423] In addition more in depth analysis of the results on respiratory function showed that effectiveness on respiratory parameters was only seen in the glucocorticosteroid-naïve patients receiving idebenone versus placebo and not in the patients receiving concomitant steroids. Importantly, respiratory parameters were higher at baseline in the steroid users compared to non-users, indicating positive effects on respiratory function by steroids itself. These analyses may indicate that the maximal treatment effect is already reached by steroids alone or that steroids suppress the effects of idebenone, although the latter seems unlikely, since there is no mechanistic explanation for this.[424] These patients have been followed for an additional two years to assess the long term safety and tolerability and the efficacy on skeletal muscle, respiratory and cardiac function, but no results have been published yet (NCT00758225). Idebenone is currently also tested in a larger group of DMD patients in two stages: first, in patients that have never been treated with corticosteroids and then in combination with corticosteroids (NCT01027884).

2.5 TNF-α and the IKK-NF-κB signalling pathway

Another hallmark of the chronic inflammation is a large increase in cytokine expression, *i.e.* TNF-α and IFN-γ.[358,425-427] TNF-α activates the IKK/NF-κB signalling pathway (fig. 2.2). NF-κB is present in all cells and is kept in an inactive form in the cytoplasm by the inhibitory protein IκB. When IκB is degraded by IκB kinase (IKK), NF-κB migrates to the nucleus where it can activate a cascade of inflammatory factors: activating pro-inflammatory macrophages and inhibiting myogenesis, thereby increasing necrosis and inflammation and reducing regeneration in muscle fibres.[428-430] Normally, anti-inflammatory factors deactivate NF-κB to reduce the inflammation. However, when NF-κB is not inactivated this can lead to chronic inflammation. In *mdx* mice NF-κB is not deactivated, but initiates a cascade of inflammatory molecules, which in turn further activate NF-κB (positive feedback loop).[431]

Since the TNF-α/NF-κB pathway is highly elevated in DMD, drugs that inhibit activation of this pathway may possibly prevent the muscle degeneration in DMD.

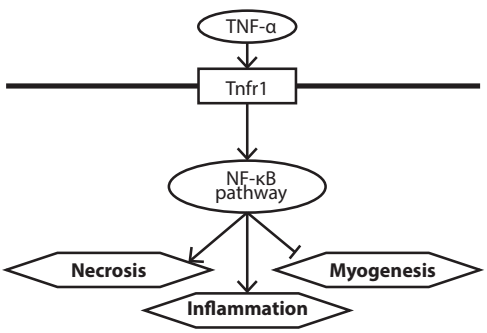

Fig. 2.2: Simplified scheme of the TNF-α/NF-κB signalling pathway
TNF-α activates the NF-κB signalling pathway, thereby increasing necrosis and inflammation and inhibiting myogenesis

Blocking TNF-α with a specific antibody (infliximab/Remicade® or etanercept/Enbrel®) has an anti-inflammatory effect and thereby reduces the breakdown of dystrophic muscle in *mdx* mice, without impairing the formation of new myotubes. This drug is already being used in other inflammatory diseases, such as rheumatoid arthritis and Crohn's disease.[392,432,433]

Pyrrolidine dithiocarbamate (PDTC) stabilises cytosolic IκB-α, thereby reducing NF-κB activity. In *mdx* mice systemic treatment with 50-75 mg/kg resulted in a decrease in muscle

fibre degradation and enhanced regeneration of skeletal muscle and diaphragm, accompanied by an improve in muscle function, and had a positive effect on the Ca^{2+}-homeostasis.[434-436] However PDTC can cause seizures at slightly higher doses (75-150 mg/kg) and brief periods of ocular discharge (excretion of fluids from the eyes) can occur already at a concentration of 50 mg/kg as used in the *mdx* experiments.[437]

NEMO-binding domain (NBD) peptide is a more selective inhibitor of NF-κB. It binds IκB-*β*, thereby preventing the formation of an IKK complex and inhibiting the activity of NF-κB. Via this pathway, NBD peptide selectively prevents inflammation-induced NF-κB activation without inhibiting basal activity required for cell survival. In *mdx* mice it greatly reduces myofibre necrosis and treatment with NBD peptide showed no dose-dependent toxicity.[428,430]

Flavocoxid, a mixed flavonoid extract with anti-inflammatory, anti-oxidant and NF-κB inhibiting properties, showed a reduced activity of NF-κB signalling pathways and so reduced muscle necrosis and enhanced regeneration in *mdx* mice.[438] A clinical trial to determine its safety in DMD patients after oral administration has been terminated for unknown reasons (NCT01335295).

Green tea extract is rich in anti-oxidants (polyphenols), the major one being epigallocatechin gallate (EGCG; a flavan-3-ol). EGCG can inhibit NF-κB activation by blocking IKK activity. Treatment of *mdx* or *mdx*^{5cv} mice with green tea extract or EGCG resulted in improvement anti-oxidant capacity, thereby decreasing muscle pathology and improving functions.[439] Treatment with green tea extract reduced fibrosis and improved muscle function in *mdx* mice.[440] Oral administration via a diet supplemented with EGCG seemed the most effective route of administration compared to subcutaneous EGCG injection.[441] A double-blinded, placebo-controlled, randomized pilot clinical trial to investigate the safety and possible effects on pathology of EGCG in DMD patients is currently ongoing (NCT01183767) and a pilot study with (-)-epicatechin (another flavan-3-ol) in BMD-patients (NCT01856868).

Another dietary derived NF-κB-inhibitor is curcumin, obtained from the spice turmeric. In *mdx* mice mixed results were observed with curcumin. Intraperitoneal injections with curcumin caused reductions in NF-κB and TNF-α levels, followed by enhancement in muscle strength.[442] In contrast, in another study, dietary supplementation with curcumin had no effect on NF-κB activity.[443] These discrepancies might be due to the different routes of administration. Studies in rats have shown the limited bioavailability of curcumin after oral compared to intravenous administration.[444]

Several other dietary derived anti-oxidant supplements (*e.g.* beta-carotene, vitamin A, vitamin C, vitamin E, and selenium) have been tested in clinical trials in DMD and other diseases. However mostly no, limited or even detrimental effects were observed.[416,445] This is probably due to lack of specific targeting. A recent study suggests increased protein thiol oxidation in dystrophic muscles plays an important role in oxidative stress mediated damage.[446] The thiol reducing anti-oxidant N-acetylcysteine that naturally occurs in vegetables reduces TNF-α and thereby NF-κB activation. After systemic treatment lower TNF-α levels were observed in *mdx* mice as well as protection against myonecrosis.[447,448] N-acetylcysteine treatment also resulted in a reduction of ROS in muscle and a decrease in centrally located nuclei. Furthermore an increase in DGC-associated proteins and utrophin expression was observed.[449]

2.6 Nutritional intervention and dietary-derived compounds

Malnutrition is commonly observed in end stages of DMD, due to eating difficulties and weight loss as a consequence of muscle wasting. Approaches to manage this are food texture modification and supplementary feeding. Importantly, daily calcium and vitamin D supplementation is recommended in combination with corticosteroid therapy, since corticosteroids can enhance osteoporosis.[450] In addition, other nutritional supplements might be beneficial against muscle wasting as well. Some dietary-derived compounds, used for their anti-oxidant properties, have been described above (see paragraph 2.4). Next to these, amino acid supplementation (taurine, creatine, arginine and glutamine) is tested in DMD patients and *mdx* mice.

Taurine is a free amino acid abundant in skeletal muscle having anti-oxidant and anti-inflammatory effects and modulating Ca^{2+}-homeostasis. Taurine deficiency has been observed in dystrophic *mdx* muscle.[451] In *mdx* mice taurine supplementation in the food prevented exercise-induced damage in hind limb muscle and ameliorated measurements of degeneration/regeneration cycles. However no change in degeneration/regeneration was seen in the diaphragm.[452] In combination with prednisolone a synergistic effect was seen in the improvement of muscle strength and Ca^{2+}-homeostasis.[453]

Creatine monohydrate is a nutritional supplement used as an ergogenic aid by athletes. After cellular uptake CK phosphorylates creatine to phosphocreatine using ATP. Phosphocreatine serves as an energy buffer and transport vehicle. Upon energy demand, *i.e.* muscle contraction, CK reverses this phosphorylation by converting phosphocreatine to ADP to regenerate ATP. Ingestion of creatine can increase creatine and phosphocreatine levels inside muscles, thereby enhancing mainly short, intense exercise performance.[454] *In vitro* the provision of creatine to *mdx* myotube cell cultures, resulted in higher phosphocreatine concentrations, improved survival, and less Ca^{2+}-accumulation by stimulating sarcoplasmic reticulum Ca^{2+}-ATPase.[455] Total creatine levels are lower in *mdx* muscles compared to wild type. In *mdx* mice a creatine-enriched diet from birth reduced the first necrosis wave normally seen after four weeks in fast-twitch, but not in slow-twitch muscle fibres. It restored the mitochondrial respiration capacity to wild type levels.[456] Less effectiveness was observed when supplementation was started after this first wave (at three month of age), suggesting necrosis was already too advanced. Only a lowering of muscle mass towards wild type levels, but no significant increase in tetanic force, was observed. As expected it did not change other pathological features, such as centrally nucleated fibres and increased Ca^{2+}-content.[457] Several clinical trials with creatine supplementation in DMD, BMD or other muscular dystrophy patients have been conducted with moderate results.[458] Small improvements in muscle strength and daily-life activities were observed in patients with several types of muscular dystrophies.[459] However in myotonic dystrophy type 1 patients, an inherited muscle disorder in adults, no improvement was observed.[460] Three months administration to DMD and BMD patients increased voluntary muscle contractions, decreased muscle fatigue and lowered urinary collagen excretion.[461] A more extensive double-blinded, placebo-controlled, cross-over study in which DMD patients, of which half was using corticosteroids, received creatine or placebo for four months followed by a cross-over after a six week washout period, showed an improvement in motor function and improvement of body composition (an increase in fat free mass) during creatine treatment irrespective of corticosteroid use. However no improvement of daily activities was found.[462] Another trial determined phosphorus metabolite ratio (PCr/Pi), which is lowered in skeletal muscle of DMD patients and associated with impaired

muscle function. In the placebo group a reduction in PCr/Pi ratio was observed, whereas an increase was seen in creatine-treated patients. Furthermore therapeutic variability was seen with age. Before treatment, PCr/Pi ratios were lower in young patients (under seven years) compared to older patients (above seven years). Whereas young creatine-treated patients showed an increase in PCr/Pi ratio after supplementation, no difference was observed in older creatine-treated patients. Some improvement/preservation of muscle strength was observed by creatine treatment.[463]

Glutamine is the most abundant free amino acid in body and muscle protein synthesis. Studies on the mechanisms behind possible anti-oxidative effects of L-glutamine have been performed in *mdx* mice, which show an increase in muscle free glutamine and glutamate, probably due to increased muscle glutamine production during catabolic stress. Exogenous administration of L-glutamine reduced the glutamine levels and decreased the ratio of oxidized glutathione versus total, which is an inducer of oxidative stress via activation of ERK1/2 that was also decreased.[464] Oral administration of L-glutamine in DMD patients for two or ten days resulted in a decrease in whole body protein degradation.[465,466] However in a longer double-blinded, randomized, cross-over trial four months of L-glutamine administration did not result in improved muscle mass or function.[467] Furthermore, although acute L-glutamine transiently stimulates insulin secretion, no effect on glucose metabolism or insulin resistance was observed.[468]

In a randomized, placebo-controlled trial comparing six month treatment with either creatine or L-glutamine versus placebo in steroid-naïve DMD patients did not improve muscle strength or function. Only in younger patients a small improvement of both compounds and in older patients of creatine might have been observed, although the difference was not significant.[469]

The activation of satellite cells (muscle precursor cells) upon damage to initiate muscle regeneration is mediated by NO-production by NOS, normally present at the sarcolemma, but mislocalised in DMD due to disruption of the DGC. L-Arginine is a substrate for NOS. Short term studies showed beneficial effects of L-arginine supplementation in *mdx* mice. After three weeks treatment of adult mice an increase in utrophin at the sarcolemma was observed.[470] This was also seen after two weeks treatment of young mice. L-Arginine led to a decrease in pro-inflammatory cytokines (IL-6 and TNF-α) which led to a decreased activation of the NF-κB pathway and its downstream targets (*e.g.* MMP-2 and MMP-9). L-Arginine treatment led to a decrease in MMP-2/MMP-9-mediated *β*-dystroglycan cleavage, thereby increasing its level. It stabilised the utrophin/*β*-dystroglycan interaction and led to targeting of nNOS towards the sarcolemma via syntrophins.[471] Also here applies that the upregulation of utrophin cannot solely be responsible for nNOS restoration,[353] but that other mechanisms must also be involved. Combinational treatment with deflazacort, which increases nNOS expression, suggested an additional benefit of L-arginine since three weeks treatment resulted in a decrease of contraction-induced injury in the limb muscle.[472] However, long term treatment with L-arginine in six months old *mdx* mice for 15 months did not affect cardiac fibrosis, although a decrease in inflammatory cell density was observed.[473] Long term studies even raised concerns of detrimental effects. Seventeen months of treatment in *mdx* mice resulted in an increase in skeletal and cardiac fibrosis. The short term effects of a decrease in MMP-2 or MMP-9 and increase in utrophin were not observed.[474] The mechanism behind this is probably a shift in macrophage type. In four weeks old *mdx* mice, at the peak of necrosis, high levels of M1 (pro-inflammatory) and M2 (alternatively-activated) macrophages were observed in quadriceps with prominent fibrosis. M1 macrophages have been shown to lyse

muscle by an NO-mediated mechanism. M2 macrophages express arginase, an enzyme that catalyses arginine into L-proline, a precursor molecule for collagen synthesis. M2a macrophages reduce lysis by M1 cells via competition of arginase with iNOS in M1 cells for its substrate arginine. At twelve weeks of age, at the peak of regeneration, an increase in IL-4 and IL-10 was observed, which deactivates M1 cells and promotes $CD136^{+}$ M2c macrophages that can increase tissue repair.[363] The deactivation of M1 cells by M2c cells can increase the substrate availability for arginase, thereby shifting the arginine metabolism from iNOS to arginase, resulting in an increase in arginase-derived metabolites, which create a more pro-fibrotic environment. Therefore L-arginine supplementation might increase arginase activity, resulting in more fibrosis on the long term. Indeed arginase-2 null mutant *mdx* mice showed reduced skeletal fibrosis; although cardiac fibrosis and function was unaltered.[474] A clinical trial assessing the safety and efficacy of L-arginine administration for 30 days on muscles, assessed by MRI, in boys with DMD or BMD, has been completed, but no results have been published yet (NCT01388764).

Long term (six months) treatment of three months old *mdx* mice with arginine butyrate did result in increased grip strength and reduced fibrosis. However no effect on skeletal muscle or cardiac histology, cardiac function and behaviour of the mice was observed.[379] Another study administering arginine butyrate for only six weeks to eight weeks old *mdx* mice also revealed an improvement in histology of the diaphragm and hind limb muscles. In addition an increase in utrophin was observed.[475] The beneficial effects might be due to the butyrate moiety that has HDAC-inhibitory activity. In the arginine butyrate-treated mice changes in HDAC-related gene expression were seen: an increase in growth promoting pathways, *e.g.* IGF-1, and a decrease in genes associated with fibrotic pathways.[379]

Payne *et al.* tested several compounds alone or as a cocktail in exercised *mdx* mice: creatine monohydrate, conjugated linoleic acid, α-lipoic acid and *β*-hydroxy-*β*-methylbutyrate. Conjugated linoleic acid consists of a number of different isomers of the essential fatty acid linoleic acid conjugated by double bonds. It can decrease body fat accretion, improve immune function and act as a free radical scavenger. A-lipoic acid is a disulfide mitochondrial coenzyme and a potent anti-oxidant that can improve mitochondrial function and enhance creatine monohydrate uptake. B-hydroxy-*β*-methylbutyrate is a leucine metabolite that is used by athletes to enhance strength and muscle mass gain by strength exercise and reduce exercise-related damage and muscle protein breakdown. Each compound separately showed some benefit and the combination of all four components showed the most prominent effect. Especially in combination with prednisolone treatment therapeutic effects were seen by increasing grip strength performance and improving muscle histology, as reflected by a decrease in centrally located nuclei and retroperitoneal fat pad stores.[476] B-hydroxy-*β*-methylbutyrate has also shown in an *in vitro* screen to improve tetanic force in dystrophic myoblasts.[477]

2.7 The TGF-*β* superfamily

The transforming growth factor-*β* (TGF-*β*) superfamily consists of proteins (myostatin, TGF-*β*, activin and BMP) involved in the regulation of many cellular processes, like cell growth, differentiation and maintenance of homeostasis. Most members are synthesized as propeptides, which become activated by proteolytic cleavage and form functional dimers. They signal by inducing and binding to receptor complexes consisting of a type II and a type I

receptor, leading to activation of downstream, intracellular signalling pathways. The affinity for type I and/or type II receptors in different cell types is affected by the presence or absence of coreceptors.[478] These ligands can signal both via a canonical Smad-dependent pathway and via non-canonical Smad-independent pathways. The Smad-dependent pathway involves the phosphorylation of Smad-proteins, which are transported to the nucleus where they regulate transcription. Myostatin, TGF-β and activin commonly use pSmad2/3 and BMP uses pSmad1/5/8.[479] Non-canonical pathways are, among others, the PI3K/Akt/mTOR, Raf/MEK/ERK and p38/MAPK pathways.[480]

Myostatin (growth and differentiation factor 8) negatively regulates muscle growth. It is present in skeletal muscle cells and in adult animals myostatin has an inhibitory effect on satellite cells. It is encoded by the *MSTN* gene located on chromosome 2q32.2 and consists of three exons. It is transcribed as a precursor protein and acquires its active form of 12.5 kDa after several posttranscriptional modifications, among which proteolytic cleavage from its propeptide.[481] Myostatin inhibits myogenesis and increases fibrosis via several pathways (fig. 2.3a). It binds mainly to the activin receptor type IIb (Acvr2b) and to a lesser extent to Acvr2a. Thereafter this complex combines with activin type I receptors Acvr1b (ALK4) or

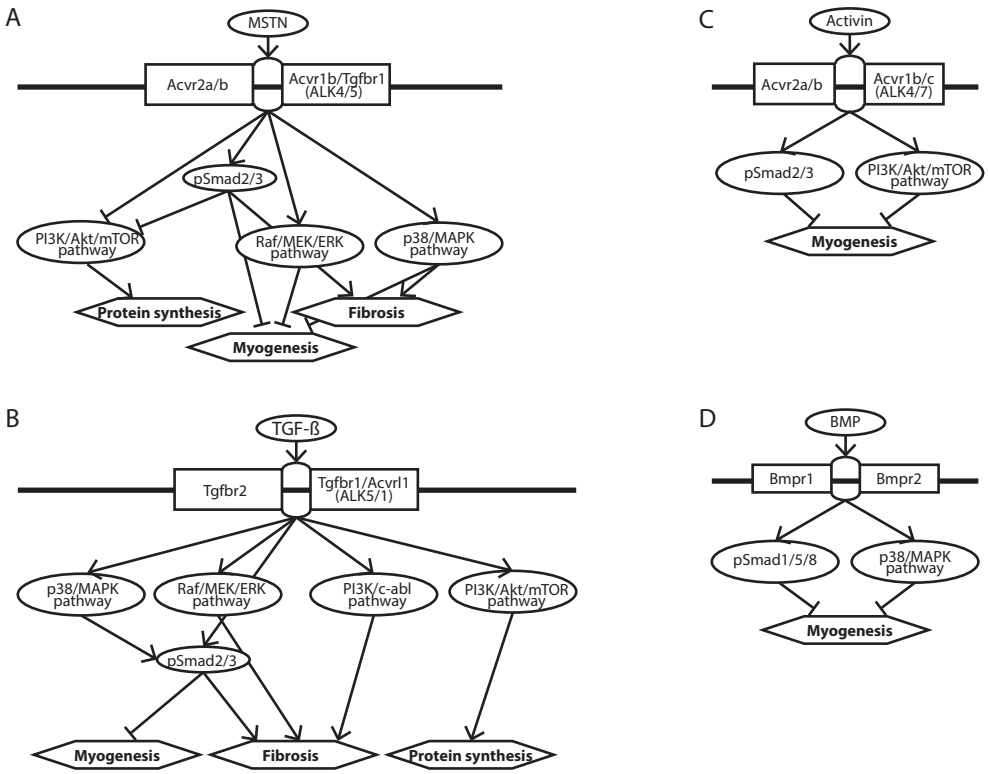

Fig. 2.3: Simplified scheme of signalling by members of the TGF-β superfamily
All members bind to type II-type I receptor combinations, leading to activation or inhibition of several intracellular pathways. They signal both via canonical pSmad-dependent and non-canonical pSmad-indepedent pathways. Myostatin (a) by signalling via pSmad2/3 and several non-canonical pathways mainly inhibits muscle growth. TGF-β (b) also signals via pSmad2/3 and various non-canonical pathways and is a potent inducer of fibrosis. Activin (c) is involved in the regulation of muscle mass via the canonical pSmad2/3 pathway and uses, amongst others, PI3K for non-canonical signalling. BMP (d) uses the pSmad1/5/8 and p38/MAPK pathway to inhibit myogenesis.

Tgfbr1 (ALK5) to activate downstream targets. Myostatin shows cell-type specific utilization of type I receptors: in myoblasts mainly the Acvr1b receptor is used, whereas in fibroblasts mainly the Tgfbr1. This is due to the presence of the coreceptor Cripto in myogenic cells, which is absent in non-myogenic cells. Cripto inhibits activin activity, which also uses the Acvr1b receptor. In non-myogenic cells myostatin uses betaglycan as a coreceptor.[482] Via the canonical pSmad2/3 pathway myostatin signalling leads to a downregulation of myogenesis transcription factors, decreasing muscle progenitor cells proliferation and differentiation. It also acts on non-canonical p38 MAPK and ERK1/2 signalling to inhibit expression of factors, *e.g.* Pax7, which leads to a decrease in satellite cell self-renewal, thereby reducing regeneration. In addition, myostatin inhibits the PI3K/Akt/mTOR pathway, both directly and via pSmad2/3, thereby reducing protein synthesis.[481,483,484] Finally, via among others pSmad2/3 and p38 MAPK it increases fibroblast proliferation.[485]

TGF-βs are another subfamily, which consists of three mammalian isoforms: TGF-β_1, TGF-β_2 and TGF-β_3. Their expression is induced in damaged and regenerating skeletal muscle and TGF-β_1 is capable of inducing fibrosis. TGF-βs bind to the type II receptor Tgfbr2, which then combines with a type I receptor, mainly Tgfbr1 or otherwise Acvrl1 (ALK1), and play a role in regulation of muscle growth, regulation and can stimulate fibrogenesis.[478,486,487] Signalling occurs both via canonical Smad2/3-dependent signalling pathways and non-canonical Smad-independent pathways (fig. 2.3b). Non-canonical TGF-β_1 signalling is mediated via pathways such as PI3K/Akt/mTOR and Raf/MEK/ERK, which all have, amongst others, pro-fibrotic effects upon activation.[480]

Activins are homo- or heterodimers of two subunits, A (inhibin-βA), B (inhibin-βB), C (inhibin-βC) or E (inhibin–βE).[487,488] They initiate signalling by binding to Acvr2a or Acvr2b, in combination with type I receptor Acvr1b or Acvr1c (ALK7) and also use pSmad2/3 for downstream signalling via the canonical pathway (fig. 2.3c). Activin A is also involved in regulation of muscle mass, since a mouse model carrying targeted deletion of inhibin-βA subunits, the constituents of activin A, displays increased muscle mass.[488] Inhibins are dimers of an inhibin-α subunit with an inhibin-βA subunit (inhibin A) or an inhibin-βB subunit (inhibin B). They inhibit activin signalling by binding to its receptors.[488]

Bone morphogenic proteins (BMPs) are known to inhibit myogenic differentiation via activating pSmad1/5/8 and p38 MAPK signalling pathways, which in turn decrease the expression of myogenic regulators like MyoD and Myog (fig. 2.3d).[489] Interestingly, expression of several BMPs was found to be increased in *mdx* muscle.[490] In DMD patient cells BMP4 was increased, which showed to inhibit myogenic differentiation.[491] The implication of induced BMP signalling and involvement in DMD pathology is currently unknown. However, recent studies showed the importance of BMPs in the regulation of myogenic differentiation. BMP type I receptor BMPR1A (ALK3) is expressed in activated satellite cells, which enhances proliferation by inhibiting genes associated with differentiation. Knockdown of the BMP antagonist Noggin enhanced proliferation, while overexpression induced premature differentiation.[492] Also in foetal muscle Noggin overexpression caused a reduced number of satellite cells and smaller muscle size.[493] After muscle injury BMP is activated and Noggin overexpression perturbed the regeneration process, leading to smaller regenerated fibres.[492,493] Recently two papers were published, which highlighted the importance of BMP signalling in stimulating muscle growth and maintaining muscle mass and the effects of interfering with BMP signalling in models for denervation-induced atrophy.[494,495] Winbanks *et al.* showed that by increasing BMP ligands or using a constantly active receptor skeletal muscle hypertrophy was promoted via Smad1/5/8-mediated activation of mTOR

signalling. Furthermore increasing BMP signalling protects muscle from atrophy during denervation, while inhibition worsens atrophy.[495] The same was shown by Sartori *et al.*, who also saw muscle atrophy after BMP inhibition. In addition BMP inhibition neutralizes the hypertrophic phenotype in mice lacking myostatin, suggesting that increased BMP signalling is responsible for the increase in muscle mass in myostatin knockout mice.[494] Therefore, BMPs are likely important regulators of myogenic differentiation, regeneration and muscle fibre growth, although the role of individual BMP ligands and receptors and their potential involvement in DMD pathology remains unknown.

Since various members of the TGF-β family and/or their receptors have shown to be differentially expressed in DMD and are involved in regulation of muscle growth and fibrosis, they serve as a target for several therapeutic approaches.

2.7.1 Myostatin inhibition

Mutations that lead to complete loss of myostatin are viable and have been described for several animals (*e.g.* Belgium Blue cattle, Texel sheep and dogs) and one human boy.[496-503] In each case lack of myostatin results in a vast increase in muscle mass. In whippet dogs that are heterozygous for a *MSTN* mutation (*i.e.* have only one functional copy) a lesser degree of muscle hypertrophy is observed, but they are more muscular and faster than wild type whippet dogs, indicating the mutation is quantitative and loss of one allele can improve skeletal muscle performance.[504] *Mdx* mice that also lack myostatin (*mdx*/*Mstn*$^{-/-}$) are more muscular and stronger than normal *mdx* mice. The muscle hypertrophy is due to an increase in muscle fibre number (hyperplasia) and in cytoplasmic volume (hypertrophy) rather than a change in the total number of myonuclei per fibre.[481] The fibrosis in the diaphragm, which increases with age, is reduced in these mice as well.[505] Furthermore, the effect of myostatin is only seen in skeletal muscle; the heart of myostatin knockout mice (*Mstn*$^{-/-}$) is similar to that of wild type mice. As anticipated, the absence of myostatin has no beneficial effects on cardiac muscle mass or cardiac fibrosis in the double knockout *mdx*/*Mstn*$^{-/-}$ mice.[506] Furthermore studies on *Mstn*$^{-/-}$ mice revealed that the increase in muscle mass and fibre size does not result in an increase in specific force, but even impairs it.[507] To study the effect of *Mstn* knockout after muscle maturation, *Mstn*$^{lf/lf}$ mice with a tamoxifen-inducible transgene have been generated to achieve muscle specific knockout in adult stages. This also leads to an increase in muscle mass, although to a lesser extent than in *Mstn*$^{-/-}$ mice.[502] So, prenatally myostatin acts predominantly on muscle progenitors and postnatally on differentiated muscle.[481] The increased muscle mass observed in all myostatin null animals served as the rationale to try to inhibit myostatin and thereby improve muscle mass in order to compensate for the loss of muscle tissue in DMD.

Treatment of C2C12-cells with pharmacological antibodies that block myostatin caused a decrease in the phosphorylation of Smad2/3, which in turn affects the formation of Smad-complexes, involved in the transcriptional regulation of genes associated with muscle cell progenitors. *In vivo* systemic (intraperitoneal) treatment of *mdx* mice with these myostatin blocking antibodies induced an increase in muscle mass and strength.[509] Importantly, a subsequent study observed improvement in diaphragm pathology by anti-myostatin antibodies in young *mdx* mice, but not in adult *mdx* mice, which already show marked disease progression.[510] Intravenous anti-myostatin antibody (MYO-029) treatment in adult neuromuscular disorder patients (including BMD patients) did not lead to a significant increase in muscle

mass in a first clinical trial. It should be noted that this was mainly meant as a safety trial: patients were treated for only one month and due to a lack of power of the study, no reliable conclusions could be drawn of the therapeutic effects. Overall the antibody was well tolerated and safe. Only at higher concentrations of ten and 30 mg/kg it caused hypersensitivity of the skin, but it had no adverse side effects on muscles. However, the hypersensitivity might limit the dose that can be used and therefore limit the therapeutic efficacy.[511]

Meanwhile, a new fusion protein joining a human antibody Fc-receptor and the ligand binding domain of type II receptor Acvr2b has been generated (sAcvr2b-Fc; ACE-031). This receptor can bind myostatin, but also its family member activin, also involved in the regulation of muscle mass and implicated in fibrosis formation in many diseases, including DMD. Thus, ACE-031 may act through separate mechanisms simultaneously, both increasing muscle mass and inhibiting fibrosis. In wild type mice it caused an increase in muscle mass, without altering the fibre type profile of the muscle, which is changed in *e.g.* myostatin deficiency.[512] In *mdx* mice similar compounds (a soluble form of Acvr2b linked to a murine Fc-receptors; RAP-031 or sAcvr2b) resulted in a normalisation of muscle force.[513,508] In a phase I clinical trial in healthy volunteers (postmenopausal women) treatment was well tolerated for both single and multiple doses and resulted in a dose-dependent increase in muscle mass at the cost of fatty tissue. Recently, a dose-escalation safety trial and its extension study in DMD patients have been terminated due to unexplained nose and gum bleeding and dilated small blood vessels observed in some treated patients (NCT01099761/ NCT01239758). The mechanisms behind these unexpected side effects are currently under investigation.[515] Combination of this sAcvr2b-Fc with AAV-U7-mediated dystrophin exon skipping resulted in increased muscle growth in *mdx* mice, as was also seen by sAcvr2b-Fc alone, and improved specific force and resistance to contraction induced injury, also seen by AAV-U7 treatment alone. No clear synergistic effect of the combination was observed.[516] In an earlier study in which the Acvr2b was knocked down by using shRNA, this knockdown strongly enhanced the force increase by AAV-U7-mediated dystrophin exon skipping. On the other hand no increase in muscle growth was observed.[517] This discrepancy might be due to the different ways of Acvr2b blockade.

As mentioned above, myostatin is synthesized as a precursor protein that is cleaved by BMP-1/tolloid metalloproteinases to generate the active C-terminal part and an N-terminal myostatin propeptide. This propeptide can inhibit myostatin activity by preventing its binding to its receptor. Myostatin propeptide fused to IgG-Fc to prevent cleavage by BMP-1/tolloid proteases resulted in increased body and muscle mass and improved function in *mdx* mice.[518] For long term expression, this fusion propeptide was delivered with an AAV8 vector, resulting in improved muscle histology, decreased CK levels and improvement of muscle force (grip strength); however it reduced exercise endurance (treadmill running).[519] To get stable transgene expression, which may not be feasible with skeletal muscle delivery, Morine *et al.* used a AAV-mediated expression of a dominant negative myostatin propeptide paired with a liver-specific promoter, thereby getting long term secretion by the liver resulting in body-wide expression. In *mdx* mice this approach resulted in increased skeletal muscle mass and function; however in 11 months old mice no improvement in diaphragm pathology was seen.[520]Administration of this same liver-targeting myostatin inhibiting compound in GRMD dogs resulted in increased muscle mass over a period of 13 months and a reduction of CK levels and fibrosis. Cardiac morphology was not assessed.[521]

Follistatin is a myostatin-ligand binding protein that can inhibit its activity. Transgenic overexpression of follistatin in mice results in a massive increase in muscle mass and increases

muscle regeneration.[522,523] Conversely, mice heterozygous for follistatin (*follistatin*$^{+/-}$) show a reduced muscle mass and regeneration.[488] An additional effect of follistatin knockout is seen in *Mstn*$^{-/-}$ mice and the effect of heterozygosity for follistatin is maintained by crossing with *Mstn*$^{-/-}$ mice, indicating that also other factors play a role in the control of muscle growth in parallel with myostatin and that follistatin also affects other ligands apart from myostatin.[488,524] Indeed, follistatin can also antagonise activin. Mice mutated for activin A also show a (more moderate) increase in muscle mass, suggesting both myostatin and activin A are involved in muscle mass regulation.[488] Administration of follistatin overexpressing muscle progenitor cells to immunodeficient *mdx* mice resulted in enhanced muscle regeneration.[522] A single injection with an AAV vector carrying a follistatin isoform that mainly affects skeletal muscle (FS-334) resulted in improvement of muscle mass and strength up to over two years in both young and aged *mdx* mice.[525] Yet, it is not selective for myostatin, but also binds to activins and inhibits their activity. Since activins have numerous functions in other tissues than skeletal muscle, increasing myostatin by using follistatin is likely to have many side effects. In *mdx* mice a transgenic follistatin-derived myostatin inhibitor FS I-I, which is much more selective for myostatin, increased skeletal muscle size and strength and reduced the infiltration of immune cells, without affecting activin activity.[526] The safety and efficacy of the FS-334 isoform expressed by AAV1 has been tested in nonhuman primates (cynomolgus macaque monkeys), resulting in increased muscle mass and strength, without immune response or toxic side effects on other organs in the long run (over one year).[527] A clinical trial where the follistatin gene (FS-334) in an AAV is injected in quadriceps muscles of BMD and sporadic inclusion body myositis (an inflammatory, progressive muscle wasting disease) patients is currently ongoing for testing safety and assessing its effect on muscle mass and function (NCT01519349). Recently combinational treatment with this FS-334 isoform and microdystrophin (ΔR4-23/ΔCT) delivery by AAV vectors was tested in aged *mdx* mice. Whereas both treatments by itself were not capable of fully protecting muscle against contraction-induced injury in this advanced disease state, the combination improved this to near normal levels as seen in wild type mice.[528]

Myostatin knockdown has also been achieved by exon skipping. 2OMePS against exon two, resulting in a premature stop codon, resulted in good exon skipping *in vitro* and lowering of myostatin expression and its downstream targets Myf5 and Myog. Also in *mdx* mice exon skipping was observed after intramuscular injection, albeit at low levels. Furthermore, in contrast to *DMD* exon skipping, which was homogenous throughout the muscle, variation in skipping levels throughout the muscle was observed. *In vitro* the feasibility of combined exon skipping of dystrophin and myostatin was shown in DMD patient-derived cells. However this has not been tested *in vivo*.[529] High levels of exon skipping were observed with PMO AONs against exon two of myostatin. Systemic injection of vivo-morpholinos induced myostatin exon skipping resulting in an increase in muscle mass and myofibre size in wild type mice.[530] Also pPMOs showed high efficiency and intramuscular treatment of *mdx* mice resulted in an increase of tibialis anterior muscle weight. When combined with a pPMO against dystrophin exon 23, both this increase in muscle mass and dystrophin exon skipping was observed, showing no detrimental effects of combining both AONs. Interestingly the increase in muscle mass after myostatin skipping only or after combined treatment was observed only in female mice, not in male mice, showing gender-specific results.[531]

Next to downregulation of myostatin signalling by exon skipping of myostatin itself, AON-mediated exon skipping has been tested for its type I receptors (Acvr1b/Tgfbr1), which

also influences other members of the TGF-β family (activin and TGF-β). Acvr1b is a receptor for myostatin and activin, and Tgfbr1 is a receptor for myostatin and TGF-β. They commonly use the pSmad2/3 pathway as one of their downstream signalling pathways. Upregulation of some of these receptors has been found in DMD patients and animal models. The mRNA coding structure of these receptors is similar. Exon two encodes the ligand binding domain and exon six part of the kinase domain; skipping of exon two will result in a non-functional receptor, while skipping of exon 6 will lead to an out-of-frame transcript. Attempts to develop AONs to skip these exons resulted in effective AONs, both *in vitro* and *in vivo*, against exon six of Acvr1b and exon two of Tgfbr1, which showed specific skipping in the targeted receptor. 2OMePS AONs against Acvr1b, but not against Tgfbr1, showed *in vitro* downregulation of myostatin induced pSmad3 levels only in myogenic cells and not in non-myogenic cells; whereas in non-myogenic cells this was only observed after Tgfbr1 skipping. This is in line with previous results showing cell-type specific utilization of type I receptors by myostatin.[482] As expected, TGF-β signalling was only affected by Tgfbr1 skipping. Enhanced myoblast and delayed fibroblast differentiation was observed after skipping with either AON. Intramuscular injection in *mdx* mice of 2OMePS AONs confirmed target specificity and showed no interference with *DMD* exon 23 skipping after co-injection. Gene expression and protein analysis also showed the different roles of Acvr1b and Tgfbr1 in disease pathology. Skipping in either receptor resulted in an increase in Myog expression, indicative of enhanced muscle differentiation. In contrast, Tgfbr1 skipping resulted in a decrease in all fibrotic markers (Collagen1a1, Serpine1 and CTGF); whereas Acvr1b skipping resulted in a decrease in Collagen1a1, but an increase in Serpine1 and CTGF. In general a reduction in pSmad2 levels was seen, suggesting an overall reduction of myostatin/activin/TGF-β signalling [Kemaladewi *et al.*, unpublished results].

A potential risk for using myostatin signalling inhibition strategies is to induce/increase DMD cardiomyopathy. Several studies have been performed on the effect of myostatin knockout/inhibition on the heart showing inconsistent results. Results in *Mstn*$^{-/-}$ mice suggest it might induce cardiac hypertrophy and thereby be beneficial for the repair of damaged cardiac muscle, although others found no cardiac phenotype.[506,532] In *mdx* mice also variable results were seen: whereas no cardiac changes were observed in *mdx*/*Mstn*$^{-/-}$ mice and mice treated with soluble Acvr2b treatment for four months, cardiac hypertrophy resulting in reduced cardiac function was observed by liver-targeted myostatin propeptide administration after eleven months.[506,519,520]

2.7.2 Targeting TGF-β signalling

Treatment of *mdx* mice with a TGF-β_1 neutralizing antibody for six weeks resulted in a decrease in TGF-β_1 expression and reduced fibrosis in the diaphragm; however no effect on muscle degeneration/regeneration was observed. Furthermore, an increase in inflammatory CD4$^+$ lymphocytes was detected. It is known that TGF-β_1 also plays a role in inflammation and immunomodulation. The overall effect of TGF-β_1-inhibition on fibrosis and inflammation could have implications for long term treatment with TGF-β_1-inhibitors and therefore combination with immunosuppressive agents might be a good strategy.[533]

Halofuginone (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) has anti-fibrotic activity by inhibiting Smad3 phosphorylation downstream of TGF-β signalling, thereby attenuating collagen type I gene expression by fibroblasts. In *mdx*

mice it could prevent an age-dependent increase in fibrosis in young mice as well as induce a decrease in fibrosis in older mice (eight to nine months old). Thereby it improved proliferation of muscle cells and skeletal muscle, diaphragm and, importantly, cardiac function.[534,535] More specifically, in *mdx* mice and muscle biopsies of DMD patients the expression of the gene collagen triple helix repeat containing 1 (*CTHCRI*) was shown to be elevated, correlating with disease severity. Large infiltrates of myofibroblasts were the source of Cthcr1 and halofuginone inhibited both these infiltrates and lowered Ctchr1 expression in skeletal and cardiac muscle of *mdx* mice.[536] A first, open-label clinical trial assessing the safety and pharmacokinetics of HT-100 (halofuginone hydrobromide delayed-release tablet) by comparing several single and multiple doses is currently ongoing (NCT018475730).

Decorin, a small leucine-rich proteoglycan, is a component of the ECM of all collagen-containing tissues. It can bind TGF-β, thereby blocking its activity. It is present in the ECM surrounding muscle fibres. Studies on DMD biopsies and cultured dystrophic myotubes found a decrease in decorin mRNA expression, mainly localised in fibroblasts, accompanied by an increase in TGF-β_1 expression.[537,538] Puzzling, another study found contradictory results, whereby decorin expression was increased in DMD biopsies, hypothesizing an attempt of the muscles to combat fibrosis.[539] Patients of the studies were in the same age range; however the last study only looked at two patients. Intraperitoneal treatment of *mdx* mice with decorin resulted in a 40% decrease of collagen I mRNA expression in the diaphragm.[540] In injured skeletal muscle decorin could prevent TGF-β_1-induced differentiation of myogenic cells into fibrotic cells.[541] Further studies on overexpression of decorin by gene transfer *in vitro* in cultured murine muscle cells showed an accelerated differentiation into myotubes and an increased expression of myogenic genes together with a decrease in TGF-β_1 and myostatin expression. *In vivo* an enhanced regeneration and healing of muscle after injury was observed by decorin gene transfer. The mechanisms behind this effect of decorin on muscle healing are not exactly known. This study suggest that it acts on various pathways, both by inhibiting fibrosis by lowering TGF-β_1 and myostatin expression and it may also upregulate regeneration, as they showed an increase in regeneration-stimulating genes like follistatin and MyoD.[542]

Suramin, a polysulfonated naphthylurea, is a TGF-β_1 blocker that was able to attenuate exercise-induced fibrosis and elevation of CK in six months old *mdx* mice after seven weeks of treatment. Only in cardiac muscle no improvement was observed.[543] This was also observed in another study treating eight month old *mdx* mice for three months. Hereby solely the heart was analysed by electrocardiogram and histology.[544] To protect the *mdx* diaphragm against muscle fatigue, it acted by decreasing MMP-9, involved in degradation of the ECM, activity and increasing β-dystroglycan, thereby having positive effects on the maintenance of the DGC.[545] Suramin is used for the treatment of other disorders (*e.g.* prostate cancer) however it may have side effects like polyneuropathy.[546]

Fibrinogen is a soluble acute phase protein, which is released from inflammatory sites and converted to fibrin, playing a role in fibrogenesis. In *mdx* mice it was shown that fibrinogen can bind the $\alpha_M\beta_2$-(MAC-1) receptor on macrophages, thereby inducing IL-1β expression, which in turn increases TGF-β expression and signalling. Fibrinogen can also induce collagen synthesis directly by binding to the $\alpha_V\beta_3$-integrin receptor on fibroblasts. In fibrotic areas in DMD patients and *mdx* mice fibrinogen expression is increased, indicating it as a possible therapeutic target. Indeed genetic loss of fibrinogen in *mdx* mice (*mdx/Fibrinogen*$^{-/-}$) resulted in a reduction in fibrosis and slowed down disease progression. The same could be achieved by pharmacological inhibition of fibrinogen in *mdx* mice with ancrod, a defibrino-

genating agent. A reduction in TGF-β, pSmad2 and collagen I expression was observed, as well as a decrease in pro-inflammatory cytokines (*e.g.* TNF-α).[547] Fibrinogen also plays an important role in blood clotting.[548] Interference with this function could increase the chance of bleeding. Since different domains of the protein are involved in the different functions, mutants could be made in which a single domain is disabled. In *Fib*$\gamma^{390\text{-}396A}$ mice expressing a mutant form of fibrinogen with normal clotting function, but lacking the $\alpha_M\beta_2$ binding motif, or blocking $\alpha_M\beta_2$-binding by administration of a fibrinogen/$\alpha_M\beta_2$-binding peptide, an amelioration of dystrophic pathology was observed, which was reversed in the *Fib*$\gamma^{390\text{-}396A}$ mice by injecting fibrinogen. These results underline the specific interaction of fibrinogen with the $\alpha_M\beta_2$-integrin receptor on macrophages. Indeed, also in DMD patient biopsies a co-localization of fibrinogen and macrophages ($\alpha_M\beta_2$-integrin) was observed in degenerating areas. Next to a decrease in $\alpha_M\beta_2$-mediated signalling, ancrod treatment resulted in a decreased inhibition of satellite cells, thereby enhancing regeneration.[549]

Another compound that has an effect on amongst others TGF-β signalling is imatinib mesylate, a 2-phenylaminopyrimidine derivative, which is already approved for the treatment of leukaemia. Imatinib selectively and competitively blocks the ATP binding sites of several tyrosine kinases, including c-Abl, c-Kit, and PDGF-receptors. The PI3K/c-Abl signalling route is also a non-Smad pathway mediating the TGF-β effects stimulating fibroblast proliferation. c-Abl can be activated by both TGF-β and PDGF. Two studies in *mdx* mice showed a decrease in fibrosis/necrosis and inflammation, thereby improving hind limb strength, after imatinib treatment. This was mediated by an inhibition of both fibrotic signalling mediators as c-Abl and PDGF and inflammatory markers TNF-α and IL-1β.[550,551] It also attenuated dystrophy in a more severe *mdx* model, the DBA/2-*mdx* mouse, by decreasing fibrosis.[552]

Bowman-Birk Inhibitor concentrate is a soy-derived serine protease inhibitor, which resulted in improvement in muscle pathology and function in *mdx* mice after oral administration. It did not act on proteasome activity but reduced calpain activity. A decreased expression of TGF-β_1 and pSmad2/3 was seen and a reduced myostatin activation, resulting in increased muscle hypertrophy and decreased fibrosis. Bowman-Birk Inhibitor concentrate inhibits several proteases, but no changes in other growth signalling pathways (p38 MAPK, Akt or NF-κB) were seen.[553]

2.7.3 BMP antagonists

BMP antagonists, *e.g.* Noggin, can enhance muscle differentiation and regeneration. Indeed, inhibition of BMP signalling with Noggin resulted in increased expression of regeneration markers both *in vitro* in C2C12-cells and *in vivo* in *mdx*/*Utrn*$^{+/-}$ mice. Muscle histology was improved in these mice. However these are non-selective BMP antagonists.[489] Furthermore, as described in paragraph 2.7 opposite effects were observed after denervation-induced atrophy. Hereby Noggin overexpression worsened pathology instead of ameliorating it.[494,495] These different observations demonstrate that BMP signalling has different effects on satellite cells/myoblasts and muscle fibres and that the effect of inhibition or stimulation is context dependent. In DMD a lot of muscle fibre damage and regeneration is observed, while in denervation-induced atrophy a lot of muscle mass is lost, leading to smaller and weaker fibres. However it indicates the large probability of side-effects while interfering with BMP signalling pathways.

2.8 The Renin-Angiotensin System

Activation of the renin-angiotensin(-aldosterone) system (RA(A)S; fig. 2.4) is known to play a role in the pathogenesis of, among others, cardiac fibrosis. This system has also been shown to be upregulated in muscular dystrophy. Angiotensin I is converted by ACE to angiotensin II, which in turn binds to its receptors angiotensin II receptor type 1 (AT1) and angiotensin II receptor type 2 (AT2). Via AT1 it asserts its main effects, among others increasing inflammation, fibrosis and stimulating cell proliferation.[554,555] It has pro-inflammatory actions by increasing the production of ROS which activate the NF-κB pathway.[556,557] Next to increasing inflammation, RAS also increases fibrosis via both TGF-β_1-dependent and TGF-β_1-independent mechanisms (fig. 2.3b/4). Firstly, it can upregulate the expression of TGF-β_1 directly via the NADPH oxidase/p38/MAPK pathway. Secondly, by enhancing downstream targets of TGF-β_1 signalling.[558] The RAS system is thought to act upon both the Smad-dependent and Smad-independent pathways. *In vitro* angiotensin II induces a late (24 hours) TGF-β-dependent pSmad2/3 activation. In cells lacking the *TGFB* gene or where endogenous TGF-β was blocked, angiotensin II induced rapid phosphorylation of pSmad2/3 and increased expression of the pro-fibrotic markers CTGF and collagen I. This was blocked by a specific AT1 antagonist. Furthermore, inhibition of p38 MAPK also diminished this phosphorylation indicating that angiotensin II activates the Smad pathway via AT1 and MAPK activation.[559,560] AT1 signalling is thought to also stimulate the non-canonical pathways PI3K/Akt/mTOR and Raf/MEK/ERK TGF-β independently.[561,562] Signalling via AT2 is thought to have opposite effects to AT1 signalling, *e.g.* anti-fibrotic and inhibiting growth, and there is negative crosstalk between both receptors. Therefore activation of AT2 during specific AT1 blockade may contribute to the beneficial effects on cardiac dysfunction.[262,563]

Considering the upregulation of RAS in DMD and its known pro-inflammatory and pro-fibrotic effects, several therapeutic strategies try to target this system at different levels.[555]

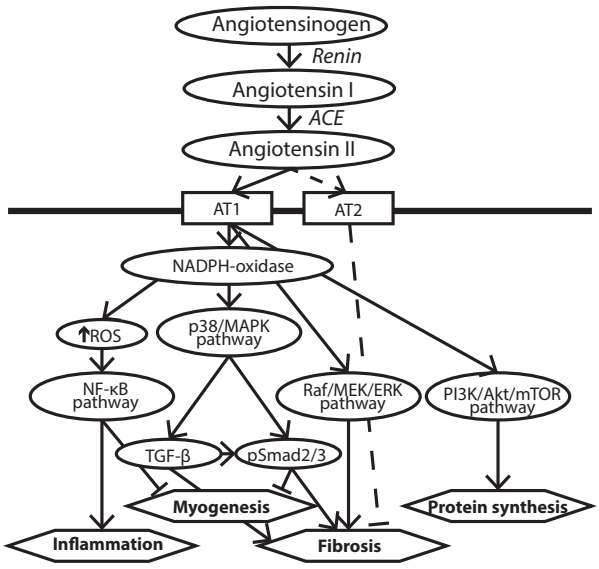

Fig. 2.4: Simplified scheme of the renin-angiotensin system
Angiotensinogen is converted by renin and angiotensin-converting enzyme (ACE) to its active form angiotensin II, which signals mainly via the AT1 receptor to increase inflammation and fibrosis. It has pro-inflammatory effects by activating the NF-κB pathway. It increases fibrosis/inhibits myogenesis both directly by activating several pSmad-dependent and independent pathways and indirectly by increasing TGF-β expression. Signalling via AT2 is thought to have opposing effects and to be induced during AT1 blockade.

The first group of compounds are ACE-inhibitors, which are already widely used in the treatment of cardiomyopathies, where they have shown to reduce morbidity and mortality. They inhibit the conversion of angiotensin I to angiotensin II by ACE and can be divided in three groups based on their molecular structure. The first group are sulphydril-containing agents (*e.g.* captopril (the first ACE-inhibitor)), the second group are dicarboxylate-containing agents (*e.g.* enalapril, perindopril and lisinopril) and the third group (phosphonate-containing agents) only includes fosinopril.[8] However the clinical relevant differences between the different groups and/or compounds are marginal.[564] Nowadays it is recommended to start ACE-inhibitor treatment, possibly combined with a β-blocker, in DMD at the first signs of cardiomyopathy.[565]

Several ACE-inhibitors have been tested *in vitro* and *in vivo* in animal models with mixed results. Cozzoli *et al.* treated young *mdx* mice systemically with enalapril and observed an improvement in muscle function, accompanied by a decrease in necrosis. More detailed analysis of biomarkers, showed an effect on expression of genes related to inflammation and oxidation (*i.e.* decrease in NF-κB and ROS), but no change in fibrotic markers (TGF-β_1 and pSmad2/3), whereupon they hypothesised that pro-inflammatory and pro-oxidative events precede the pro-fibrotic events.[554] On the contrary, Nelson *et al.* treated young *mdx* mice for a short period with enalapril and did not observe an improvement in forelimb grip strength. However these results are rather limited, since they did not measure anything else.[263] The contradicting results could also be due to the fact that mice in the first study underwent an exercise protocol to aggravate muscle inflammation and pathology, making it easier to induce improvement. Furthermore the RAS potentially plays a role in impairing exercise performance. However, recently another group observed an improvement in muscle strength and decrease in CTGF-induced pro-fibrotic activity both in sedentary and exercised *mdx* mice. Again no change in TGF-β_1 expression.[566]

Lisinopril was identified in a large *in vitro* drug screening assay in dystrophic muscle to increase tetanic force.[477] When combined with an aldosterone antagonist, spironolactone, it was able to preserve cardiac function, reflected by a decrease in cardiomyocyte damage and fibrotic markers (MMPs), in *mdx*/*Utrn*$^{+/-}$ mice. Therapeutic effects at 20 weeks of age were larger when treatment was started at four weeks of age compared to start of treatment at eight weeks of age, indicating the importance of early intervention.[567]

Captopril showed its effectiveness *in vitro* by partially counteracting TGF-β_1-induced myoblast differentiation inhibition in C2C12-cells.[568] *In vivo* captopril was able to improve cardiac function in older *mdx* mice after starting treatment at two months of age.[381]

Studies in patients have shown positive effects of several ACE-inhibitors on cardiac function. Enalapril showed normalization of left ventricular function in half of the patients. The other half of the patients did not respond to enalapril treatment, but no association with type of mutation or age of onset of cardiac problems was found.[15] ACE-inhibitor therapy is often combined with β-blockers (see paragraph 2.15). Several ACE-inhibitors (often enalapril or lisinopril) showed to delay the progression of cardiomyopathy or even improvement of heart function and improvement of long term survival, when used alone or in combination with a β-blocker in DMD patients with heart failure.[569-571] In a randomized double-blinded clinical trial with perindopril versus placebo no difference between left ventricular ejection fractions (LVEFs) was observed after 36 months of treatment. Thereafter all patients (also the placebo) received in an open-label extension study perindopril for an additional two years. After this period a higher LVEF was seen in the patients who had received perindopril for five years compared to those who only received it during the last two years. Possible reasons for the

absence of an effect after the first phase, could have been the low *a priori* risk of developing heart failure (patients had normal LVEF at baseline) and the small study population. Since a placebo group is lacking in the second phase no conclusion can be drawn for the effect of perindopril in the group that started treatment later.[572] A ten year follow-up of the same patients showed a higher survival rate in the patients who received perindopril from the start of the trial (26 out of 28 versus 19 out of 29).[573] Overall all studies indicate the importance of early initiation of treatment.[565]

A clinical trial with the anti-oxidant coenzyme Q10 (see paragraph 2.4) and lisinopril is currently ongoing (NCT01126697). This open-label trial will compare the safety and the effects on cardiac function of coenzyme Q10 alone, lisinopril alone or combined treatment in DMD, BMD and limb girdle muscular dystrophy (LGMD) patients.

A second group of compounds targeting the RAS system are selective AT1 antagonists, to which losartan belongs. Losartan is used as a vasodilator to treat hypertension and is known to decrease TGF-β signalling.

Cohn *et al.* first investigated the effect of losartan in *mdx* mice. Long term losartan treatment (from six weeks of age till six to nine months) resulted in decreased disease progression and fibrosis and improved muscle function. In addition, they showed that after muscle injury in aged *mdx* mice, losartan improved the regenerative capacity and reduced fibrosis by antagonism of TGF-β signalling, reflected by lower levels of its downstream targets pSmad2 and TSP-1.[260] However a few years later the same group published another study indicating the protective effects were not mediated via TGF-β signalling, but via the IGF-1/Akt/mTOR pathway.[262] They showed in a mouse model for sarcopenia (loss of skeletal mass and function during ageing), that losartan improved muscle architecture and functional recovery after cardiotoxin injection via blocking both the canonical (*i.e.* pSmad2) and non-canonical (*i.e.* pERK) TGF-β signalling pathway. However, experiments in disuse atrophy during ageing, showed no change in either pSmad or pERK, but positive effects by activation of the IGF-1/Akt/mTOR pathway.[561] This pathway plays a role in increasing protein synthesis and preventing muscle regeneration.[574,575] In relation to these partly contradicting results, we did not see any change in *mdx* mice in the expression of downstream targets of TGF-β signalling in fibrotic pathways, after losartan treatment. In addition no effect of losartan on pSmad2 levels after TGF-β stimulation was observed in an *in vitro* assay. Only a modest increase in pERK levels might be seen (this thesis chapter 6). In addition, in the meantime other groups published less convincing results on the positive of losartan in muscular dystrophy. Nelson *et al.* indeed observed improvement of forelimb grip strength in *mdx* mice by losartan treatment, but only at two months of age and not at nine months of age. At two months, a decrease in TGF-β activity was observed, but at nine months there was no change in fibrotic markers. However they did observe an improvement in respiratory function in these aged mice.[263] In two other studies long term (six months resp. two years) losartan treatment in *mdx* mice could only preserve cardiac function, but not skeletal muscle function. Probably the angiotensin II pathway plays a major role in the development of cardiac fibrosis, whereas in skeletal muscles other fibrotic pathways dominate.[261,264] This is in line with the results for ACE-inhibitors, whose main beneficial effects are also observed in cardiac tissue. A clinical trial comparing the efficacy of losartan to the ACE-inhibitor lisinopril in DMD patients with impaired cardiac function (ejection fraction <55%) has been conducted, but not yet published (NCT01982695).

2.9 Insulin-like growth factor 1 stimulation

Other ways to influence myogenic regeneration and differentiation are via insulin-like growth factor 1 (IGF-1) signalling. IGF-1 is a growth factor that plays a role in the maintenance of muscle mass and muscle growth. It stimulates satellite cell proliferation and differentiation during muscle regeneration via activating the PI3K/Akt/mTOR pathway, which stimulates protein synthesis and blocks apoptosis and via the Raf/MEK/ERK pathway, which increase cell growth and differentiation (fig. 2.5).[574,576,577] Therefore compounds that increase IGF-1 signalling may promote muscle hypertrophy and increase regeneration in muscle wasting disorders like DMD.

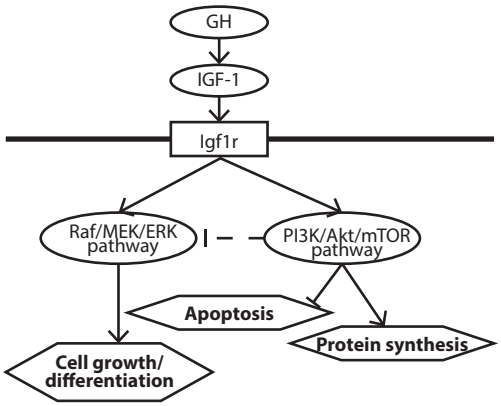

Fig. 2.5: Simplified scheme of the IGF-1 signalling pathway
Growth hormone (GH) stimulates IGF-1 production, which binds to the Igf1 receptor, thereby activating the PI3K/Akt/mTOR pathway to stimulate protein synthesis/block apoptosis and the Raf/MEK/ERK pathway to increase cell growth/differentiation.

Various IGF-1 analogues have been tested in *mdx* mice. A recombinant human IGF-1 improved contractile function of the diaphragm and fatigue resistance of hind limb muscles.[578,579] Whereas IGF-1 overexpression causes hypertrophy at a high dose (for example by transgenic overexpression in *mdx*/*Mglf*$^{+/+}$ mice),[580] at a low dose (1.0-1.5 mg/kg/day) it causes a shift of muscle fibre type towards a more oxidative and fatigue-resistance type, without changing the muscle mass. Thereby it reduces functional muscle deficits in *mdx* mice. It is known that in DMD patients fast-twitch muscles are more susceptible to damage than slow-twitch muscle.[581] A compound that releases IGF-1 from its binding peptides (IGF-1 aptamer; NBI-31772), thereby increasing the level of free IGF-1, also protected leg muscle and diaphragm to contraction-induced injury by shifting towards a slower muscle type when administered via continuous infusion (6 mg/kg/day) to *mdx* mice. However it increased muscle fatigability during repeated maximal contractions, but these are unlikely to be made by patients in practice.[582] An improved IGF-1 variant (PEG-IGF-1) showed that the benefit of treatment depended on the severity of pathology. In young (mildly affected) *mdx* mice it indeed protected skeletal and diaphragm muscles; yet these effects were less pronounced in more severely affected older *mdx* mice and absent in very severely affected *mdx*/*Utrn*$^{-/-}$ mice.[583] In a randomized clinical trial in DMD and BMD patients growth hormone, which stimulates IGF-1 production, induced a slight improvement in systolic heart function after three months, but no changes in skeletal muscle function.[584] A longer study to test the safety and efficacy of IGF-1 in DMD patients is currently ongoing (NCT01207908).

2.10 Anabolic agents

In contrast to catabolic agents, like corticosteroids, which reduce muscle fibre size, anabolic agents aim to increase fibre size. The rationale behind the first approach is that smaller fibres might be less susceptible to contraction-induced injury, thereby reducing the degeneration/regeneration cycles, whereas the idea behind the second approach is that increasing fibre size will increase muscle strength and enhance muscle repair. However, the latter might also make the muscles more susceptible to injury, thereby accelerating degeneration.[585]

B-adrenoreceptor agonists (β-agonists) are hormone-like substances that bind to the β-adrenoreceptors on the cell membrane. These receptors play a regulatory role in cardiovascular, respiratory, metabolic and reproductive functions. Skeletal muscles contain mainly β_2-adrenoreceptors. B-agonists bind to these receptors and activate a cAMP-protein kinase A signalling pathway, which is involved in protein synthesis and protein degradation. Via this pathway they have anabolic effects on skeletal muscles and can increase muscle growth and repair after injury.[586]

B_2-agonists like clenbuterol and albuterol were shown to have anabolic properties in *mdx* mice by increasing skeletal muscle mass.[587,588] Results on muscle histology and function are less consistent. It decreased muscle degeneration in both younger (20 weeks old) and old (87 weeks old) *mdx* mice and also prevented exercise-induced fibrosis after ten weeks of treatment.[588] However, 20 weeks treatment of six months old mice had no impact on force production.[587] Importantly no improvement of diaphragm muscle, either on fibrosis or force production, could be observed.[589]

Another β-agonist, albuterol, has been tested in clinical trials with DMD and BMD patients. In a pilot study 12 weeks of treatment with 8 mg/day showed only a small increase in muscle strength in DMD or BMD patients, without side effects.[590] In a longer double-blinded, placebo-control trial where patients received 12 weeks of albuterol treatment and 12 weeks of placebo treatment with 12 weeks in between, or the other way around, also mainly an increase in muscle mass was seen, which might be accompanied by an improvement in functional performance, but no increase in strength of several muscle groups.[591] The same was observed for clenbuterol in trials for facioscapulohumeral dystrophy, where mainly increases in muscle mass were observed, but only moderate or no improvements in muscle strength or function.[592-594] A small study in a few adult muscular dystrophy patients, among which one Becker patients, suggested that clenbuterol might be beneficial in early stages of disease, since it could improve better preserved muscles, but had no effect on more atrophic muscles.[595]

Clenbuterol and albuterol are older generation β-agonists. Formoterol is a new generation, more powerful β_2-agonist, which is already used for other diseases. In ten weeks old *mdx* mice, after four weeks of daily treatment with a low dose, it increased muscle mass, diameter and force producing capacity in peripheral muscle, without increasing muscle fatigue. Higher doses have generally shown to increase muscle fatigue. However also formoterol could not improve diaphragm muscle function.[596] A concern could be that increasing muscle size might increase their susceptibility to contraction-induced injury, since it has been observed that large, fast-twitch type II fibres, the most affected fibre type in DMD, are especially vulnerable to lengthening contractions. However, on the contrary, inducing muscle hypertrophy in these type of fibres by formoterol treatment in *mdx* mice did improve their force producing capacity and made them less susceptible to contraction-induced injury.[597]

A problem with the use of β-agonists can be that prolonged treatment leads to a downregulation of β-receptors on the muscle cell membrane and chronic administration is toxic for the heart and causes muscle tremor.[586] Since β_1-adrenoreceptors predominate in the heart, this might be prevented by the use of a highly selective β_2-adrenoreceptor agonists, like formoterol. Unfortunately these still lead to mitochondrial dysfunction, as reflected by a decrease in mitochondrial protein synthesis and oxidative capacity after chronic treatment of rats or mice, by reducing SR Ca^{2+}-ATPase activity in the heart and impairing cardiac relaxation.[598,599]

Anabolic androgenic steroids (AAS) are derivatives of testosterone. They have both anabolic (increasing protein synthesis and muscle growth) and androgenic (stimulating primary and secondary sexual development in males) effects. They are well known for their (ab)use by athletes because of (supposed) effects on increasing muscle mass and strength. Their anabolic effects occur via both direct and indirect mechanisms. Directly, they bind to the androgen receptor, thereby stimulating MHC protein synthesis. Indirectly, they competitively bind to glucocorticosteroid receptors, thereby blocking those signalling pathways and decreasing protein catabolism. Furthermore testosterone has shown in men to increase IGF-1 expression thereby increasing muscle protein synthesis.[600]

The AAS oxandrolone, an oral synthetic analogue of testosterone, has been tested in clinical trials in DMD patients. In a first pilot experiment an increase in overall functional muscle score was seen after three months of treatment.[601] Thereafter a larger randomized, double-blinded, placebo-controlled trial for six months was performed, with moderate results. Although some quantitative muscle tests showed an improvement, overall there was no significant improvement in muscle strength compared to the controls. Only, it may be that the oxandrolone-treated patients did not get worse, whereas the controls showed deterioration, but this was not significant.[602] A subsequent study on the mechanisms behind a possible muscle strength increasing effect of oxandrolone, showed, after three months of treatment, an increase in synthesis of MHC proteins and gene expression data suggested a decrease in muscle regeneration.[603]

Although in the oxandrolone trials no negative effects on muscle were observed or possibly even positive effects, an earlier study with another AAS, nandrolone decanoate, in *mdx* mice showed worsening of pathology. Three weeks treatment of young mice resulted in an increase in myofibre damage and CK levels.[604] The dose used in these mice (1.5 mg/kg/day) was comparable to those used in the clinical trials (0.1 mg/kg/day) after applying a correction factor based on normalisation to body surface area when translating doses between small and larger animals (1.5 mg/kg in mice≡0.12 mg/kg in humans).[605]

A possible explanation for the varying results with AAS could be due to the effects they exert on non-muscle tissues. Selective androgen receptor modulators such as GLPG0492 are non-steroidal hormones that are more selective for skeletal muscle and bone. In a comparative trial for four weeks in exercised *mdx* a high dose (30 mg/kg, 6 days/wk) resulted in increased fatigue resistance, diaphragm force and less fibrosis, whereas nandrolone (5 mg/kg) and α-methylprednisolone (1 mg/kg) only had a positive effects on some of these functions. No negative effects were observed with nandrolone. In a 12-weeks study the effect of GLPG0492 was maintained, also with lower doses (3 mg/kg or 0.3 mg/kg).[606]

Estrogens are mostly seen as female sex hormones, but skeletal muscles are a major source of estrogen production in both men and women. In muscle, estrogens can increase force output and protect them against injury. Tamoxifen is a selective estrogen receptor modulator with

anti-estrogenic effects that is used in the treatment of breast cancer. It has numerous actions, including scavenging of free radicals, inhibiting fibrosis and acting on Ca^{2+}-homeostasis; all playing a role in DMD pathology. Oral treatment of *mdx*^{5cv} mice resulted in a large amelioration of dystrophic pathology, reflected by an increase in force production and histology. Importantly, also diaphragm and cardiac fibrosis was decreased.[607,608]

2.11 HDAC-inhibitors

Histone deacetylases (HDACs) play a role in the control of signalling networks involved in among others muscle growth, de- and regeneration. NO signalling regulates activity of HDAC2, a class I HDAC, by S-nytrosylation, which inhibits HDAC2-mediated gene repression, among which the myostatin inhibitor, follistatin. NO is produced by nNOS, which activity is disturbed in DMD (see paragraph 1.2.2), consequently HDAC signalling is also likely to be deregulated. Therefore HDAC-inhibitors (small molecule drugs) may be beneficial to DMD patients, since they can promote muscle hypertrophy and regeneration.[609]

The HDAC-inhibitors trichostatin A, valproic acid and phenyl butyrate decreased CK levels in 12 weeks old *mdx* mice. Trichostatin A was shown to be most efficient, also in inducing myotube formation and increasing regeneration marker expression in cells derived from treated mice. Furthermore increased mortality was seen amongst valproic acid- and phenyl butyrate-treated animals, whereas no side effects were observed due to trichostatin A treatment. Trichostatin A treatment resulted in an improved muscle cell integrity and recovery to exercise performance by increasing myofibre size, mediated by an upregulation of follistatin, reducing fibrosis and inflammatory cell infiltrates.[610]

Another HDAC-inhibitor, givinostat, was tested long term (3.5 months) in six weeks old *mdx* mice, leading to improvement in muscle formation, histology and function.[611] Givinostat is currently tested in DMD patients in an open-label phase I/II study (NCT01761292). First the safety and tolerability of escalating doses will be assessed. If this is well tolerated, patients will be treated for a year to determine the effects on histology and functionality.

As described in paragraph 1.2.2, in addition to the DGC, the $\alpha_7\beta_1$-integrin complex links laminin in the ECM to the actin cytoskeleton. After observing that transgenic overexpression of the α_7BX2 chain extended the life span of *mdx*/*Utrn*$^{-/-}$ mice by threefold and ameliorated the pathophysiology,[612] Gurpur *et al.* searched for compounds that could increase α_7-integrin levels. Hereby they also identified valproic acid, an FDA-approved branched chain fatty acid. Next to HDAC-inhibiting activity, this is also an activator of Akt. *In vitro* valproic acid increased α_7-integrin thereby increasing hypertrophy and decreasing apoptosis via activating the Akt/mTOR/p70S6K pathway. Five weeks treatment of three months old *mdx*/*Utrn*$^{-/-}$ mice resulted in decreased fibrosis and inflammation and improvement of hind limb contractures. Detailed analysis showed an increased expression of Akt and a decreased expression of ERK. Here no increased mortality was reported although the used dosage (240 mg/kg twice daily) was higher than in the previous study by Minetti *et al.* (160 mg/kg/day).[613] In myotubes these pathways work antagonistic, whereby the Raf/MEK/ERK pathway promotes differentiation, thereby reducing hypertrophy, and the PI3K/Akt/mTOR pathway inhibits differentiation, thereby inducing hypertrophy. Both pathways are affected by valproic acid. Furthermore Akt can inhibit the Raf/MEK/ERK pathway in differentiated myoblasts, but not in their myoblast precursors.[614] Valproic acid was able to activate Akt by α_7-integrin independently, since, in

contrast to the cultured muscle cells, no change in α_7-integrin levels was observed in these mice. This discrepancy is probably due to the short serum half-life of valproic acid, therefore dosing was not optimal to have an effect on α_7-integrin.[613]

In an *in vitro* reporter gene assay using HEK293 cells, a cell line derived from human embryonic kidneys, these HDAC-inhibitors, trichostatin A and valproic acid, were shown to enhance exon skipping by several 2OMePS AONs. A small pilot study by us in cultured human control myoblasts, did not show differences in AON-induced exon skip efficiency between valproic acid treated cells and control cells [Verhaart *et al.*, unpublished results].

2.12 Improvement of calcium homeostasis

As mentioned before Ca^{2+}-homeostasis is disturbed in DMD. Controversy exists whether this is a secondary, passive process due to the increased membrane permeability or a direct effect of defects in Ca^{2+}-regulating mechanisms.[615] Both hypotheses do not have to be mutually exclusive. The absence of dystrophin and thereby disruption of the DGC leads to damage of the sarcolemma, increasing its permeability. Hereby the influx of calcium is increased. At first, compensatory mechanisms can account for this increase influx, but eventually this capacity is exhausted and Ca^{2+}-homeostasis is lost. The high Ca^{2+}-concentration leads to activation of calpains (Ca^{2+}-dependent proteases), causing cell and membrane proteolysis, in turn increasing membrane damage and Ca^{2+}-influx, eventually leading to cell death.[616] On the other hand, it might also be a direct effect of a higher activity of Ca^{2+}-channels and defects in proteins involved in the removal of intracellular calcium.

Stretch-activated channels for non-specific cations (SAC_{NSC}) allow the entry of calcium and sodium into cells under normal conditions. Studies have shown that these channels are more active in *mdx* mice, where both resting and stretch-induced intracellular Ca^{2+}-concentrations are elevated compared to wild type mice. This was prevented by blocking of these SAC_{NSC}, which also decreased membrane permeability, suggesting this to be a secondary effect of increased Ca^{2+}-influx. As described above oxidative stress occurs in DMD and ROS are increased. Studies suggest that ROS contribute to the activation of kinases that open these Ca^{2+}-channels.[617] Next to SAC_{NSC}, also others channels have shown to be deregulated in DMD and/or *mdx* muscle fibres. TRPV2, a cationic channel with mechanosensitivity, is present in elevated levels at the sarcolemma of dystrophin-deficient muscle fibres. Transgenic expression of a dominant-negative TRPV2 mutant in *mdx* mice improved muscle histology and performance.[618] Furthermore, store-operated calcium entry (SOCE) is increased, which is influenced by the Ca^{2+}/PLC/PKC pathway and regulated by the scaffolding protein α1-syntrophin, which is associated with the DGC.[619]

Next to an increase in Ca^{2+}-influx, an impaired removal might also contribute to the elevated intracellular levels. A protein involved in this process, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase was found to be dysfunctional in *mdx* and *mdx*/*Utrn*$^{-/-}$ mice.[620] In addition to the activation of proteases, the disturbed Ca^{2+}-homeostasis also contributes to the pathophysiology by increasing mitochondrial permeability, causing swelling of the mitochondria and downregulation of mitochondrial genes (mitochondrial permeability transition). Over time this causes rupture of the mitochondria, resulting in a metabolic crisis and contributing to apoptosis/necrosis of the cells. These processes might be regulated by cyclophilin D, a mitochondrial matrix prolyl cis-trans isomerase, encoded by the gene *Pfif*. The *Scgd*$^{-/-}$ mouse (lacking δ-sarcoglycan) is a model for severe dystrophy in skeletal mus-

cle and heart. Knockout of the *Pfif* gene in these mice prevents mitochondrial swelling and thereby myofibre necrosis.[621] In addition, Ca^{2+}-dependent signalling transduction pathways are stimulated, which, through negative feedback, lead to a downregulation of these pathways, thereby contributing to the metabolic crisis.[622]

Pentoxifylline, the 1-5-oxohexyl analogue of the methylxanthine theobromine, is a compound with anti-inflammatory, anti-oxidant properties and effects on Ca^{2+}-homeostasis. It inhibits human dermal fibroblast proliferation and synthesis of, among others, collagen by these cells by selectively blocking the induction of collagen synthesis by TNF-α.[623,624] Furthermore it increases cAMP levels by inhibiting phosphodiesterase, thereby reducing the activity of Ca^{2+}-channels, which improves Ca^{2+}-homeostasis.[625] Studies of effects of pentoxifylline in *mdx* show mixed results. Pentoxifylline treatment (50-100 mg/kg) resulted in an improvement of muscle histology and muscle strength.[401,625] Another study showed a small delaying effect of pentoxifylline (150 mg/kg) on necrosis and increased mechanical function and resistance to fatigue of skeletal muscle.[439] However, Gosselin *et al.* reported that a lower dose of pentoxifylline (16 mg/kg) had no effect on collagen in the diaphragm muscle and it also did not improve its contractile function.[626] Subsequent clinical trials in DMD patients showed disappointing results. In an open-label safety study, pentoxifylline treatment for one year via an orally administered immediately release formulation was so poorly tolerated (65% of the patients experienced intolerable gastrointestinal side effects) that half of the patients withdrew from the trial and no conclusions of its efficacy could be made.[627] To circumvent these gastrointestinal side effects a subsequent randomized, double-blinded trial used a slow-release formulation. Yet, mild to moderate gastrointestinal and hematologic adverse events were still observed. In addition, no improvement or prevention of deterioration of muscle strength or function was found.[628] Importantly an *in vitro* assay on the contractile function of dystrophic myoblast showed a complete inhibition by pentoxifylline on the positive effect seen after treatment with prednisolone and creatine, highlighting the risk of deleterious interactions of cocktails of medicines used by some patients.[477]

BGP-15 (an [*O*-(3-piperidino-2-hydroxy-1-propyl)-nicotinic acid] amidoxime derivative), a compound that increases the expression of Hsp72, was able to preserve muscle strength and decrease fibrosis in both mild *mdx* mice and severely affected *mdx*/*Utrn*$^{-/-}$ mice. Hsp72 is a protein that inhibits pro-inflammatory cytokines like TNF-α and the NF-κB pathway. However the beneficial effects of BGP-15 were likely mediated by improvement in Ca^{2+}-homeostasis and not by a decrease in inflammation, since no differences in these inflammatory markers between *mdx* mice overexpressing Hsp72 (*mdx*$^{TG(+)}$) and normal *mdx* mice were found. Further analysis showed that sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase activity was increased in these *mdx*$^{TG(+)}$ mice and that BGP-15 also stimulated its activity. BGP-15 is already used in clinical trials for diabetes.[620]

The L-type voltage-dependent Ca^{2+}-channel blockers verapamil and diltiazem were able to significantly decrease the Ca^{2+}-levels in heart muscle of *mdx* mice and there was a trend observed in the diaphragm. Degeneration of the diaphragm was decreased and thereby the dystrophic phenotype improved. Both drugs have a cardiovascular-targeted effect, so mainly act on the heart and the diaphragm. They did not decrease the Ca^{2+}-content of the skeletal muscle or even increased it.[629] A double-blinded clinical trial showed that, although after one year of treatment with diltiazem the number of Ca^{2+}-positive fibres was lower in the treated group versus placebo, after three years no obvious clinical benefits were observed.[630] Chronic treatment in another double-blinded trial suggested small beneficial effects by improving

some skeletal muscle functions and cardiac parameters, although all results were not significant.[631] Overall, several trials with these and related Ca^{2+}-channel blockers showed no beneficial effect in DMD.[632]

In *mdx* mice a structural and functional defect in RyR1, a sarcoplasmic reticulum Ca^{2+}-release channel, has been found too, which leads to 'leaky' channels.[59] NO-mediated hypernitrosylation of this channel has also been found in BMD patients with deletions in the nNOS binding region (exons 42 to 45), which resulted in increased cytosolic NO production, thereby causing disturbance of the RyR1/calsatabin-1 complex, important for Ca^{2+}-signalling. This is probably also the case in DMD, since there also nNOS is mislocalised in and NO-production disturbed.[633] Furthermore activation of RyRs has been shown to be able to evoke SOCE.[619,634] S107, a compound that inhibits these RyR1 channels, prevented Ca^{2+}-leak and thereby improved muscle function and exercise performance in *mdx* mice.[59]

Debio-025 is a drug that inhibits cyclophilin D, which is probably involved in mitochondrial dependent necrosis of myofibres. Subcutaneous treatment of both *mdx* and *Scgd*$^{-/-}$ mice (50 mg/kg) with debio-025 reduced swelling of the mitochondria and reduced fibrosis in the skeletal muscle and the diaphragm. Another cyclophilin D inhibitor is cyclosporine. However, cyclosporine is not selective for cyclophilins, but also inhibits calcineurin. Calcineurin is an important signalling protein for skeletal muscle regeneration after injury and differentiation of skeletal muscle cells and also increases the expression of utrophin (which can functionally compensate for loss of dystrophin). Thus, cyclosporine will have both a positive and a negative effect, which are likely to negate each other. By contrast, debio-025 has no inhibitory effect on calcineurin activity and is more potent in inhibiting cyclophilin D than cyclosporine.[621]

Skeletal muscle primarily expresses three isoforms of calpain called μ-calpain (calpain 1), m-calpain (calpain 2) and p94 (calpain 3). As calpain activity is increased in dystrophic muscle, calpain inhibitors may be beneficial. On the other hand, mutations in calpain 3 are the cause of LGMD type 2A (LGMD2A), indicating its important role in normal muscle functioning.[635] Therefore caution might also be required with this approach. Experiments with several calpain inhibitors showed mixed results. Calpastatin is an endogenous specific inhibitor of μ- and m-calpains. Transgenic overexpression of calpastatin in *mdx* mice resulted in decreased proteolysis by calpains. Histological benefit was seen or not, depending on which promoter was used.[636,637] Leupeptin (*n*-acetyl-L-leucyl-L-leucyl-L-argininal) is a non-specific calpain inhibitor. A short term study with intramuscular injections in *mdx* mice, showed improvement of muscle histology, reflected by decreased muscle degeneration, less centrally located nuclei and increased myofibre diameter.[638] In contrast, long term systemic treatment (six months) showed no improvements. A novel, more specific compound in which the inhibitory portion of leupeptin is linked to carnitine to improve muscle uptake (C101), also failed to improve muscle function and histology in both *mdx* mice and GRMD dogs. This is probably due to compensatory mechanisms, whereas activation of endogenous m-calpain and potentially also μ-calpain is increased in the presence of an exogenous calpain inhibitor.[608,639]

BN 82270 is a membrane-permeable prodrug of a chimeric compound BN 82204 that acts dually, both as a calpain-inhibitor and anti-oxidant, thereby targeting both damage due to disrupted Ca^{2+}-homeostasis and ROS-induced damage. In *mdx* mice it prevented calpain-overactivity in the diaphragm, thereby reducing fibrosis. Longer treatment (4-6 weeks) resulted in improved muscle function and lowering of TGF-β_1 levels in skeletal muscle and diaphragm. However, almost no improvement in histopathology of the gastrocnemius was observed.[640]

2.13 Matrix metalloproteinases

MMPs are zinc-dependent endopeptidases that play an important role in ECM degradation, inflammation and fibrosis in various pathologies. Differential expression of several MMPs has been found in DMD as well. MMP-2 and MMP-9 are downstream targets of NF-κB, which cleave the extracellular domain of *β*-dystroglycan, thereby disrupting the interaction with *α*-dystroglycan. An upregulation of MMP-2 and the natural inhibitors of MMPs, TIMP-1 and TIMP-2, was found in DMD muscle.[641] The same accounts for MMP-9, the expression of which has been shown to correlate with disease progression, *i.e.* to be higher in older DMD patients and to increase over time.[642] In *mdx* mice also an upregulation of MMPs was found, but a downregulation of TIMPs.[643] Inhibition of MMP-9 in *mdx* mice has shown to have a beneficial effect by decreasing inflammation and fibrosis. These effects are mediated by inhibition of both the NF-κB pathway and the caveolin-3 pathway.[644]

Since inhibition of MMP-9 has shown to have therapeutic effects in *mdx* mice and other MMPs that play a role in among others fibrosis, are upregulated in DMD as well, they might also serve as a therapeutic target.

Batimastat (BB-94) is an inhibitor of a broad spectrum of MMPs, including MMP-1, MMP-2 and MMP-9 and has reported to be effective in cancer models. Short term treatment of *mdx* mice resulted in a decrease in necrosis and inflammation, leading to an improved muscle function. Furthermore an improvement in components of the DGC and nNOS levels was seen.[643]

Different MMPs often activate each other, however the exact roles of different MMPs in skeletal muscle are not known. Furthermore, individual MMPs have shown to play different roles depending on the disease stage, therefore inhibition of MMPs can both be advantageous as well as disadvantageous. Although MMP-2 was shown to be upregulated in DMD, genetic knockout of MMP-2 in *mdx* mice (*mdx*/*MMP-2*$^{-/-}$) worsened pathology by impairing the growth of regenerating muscle fibres through reduction of VEGF-mediated angiogenesis.[645] In addition, although MMP-inhibitors as batimastat and marimastat have been found to be beneficial in several types of cancers and are used in cancer clinical trials, no successful results have so far been shown in these trials due to side effects. Therefore global inhibition of MMPs could be deleterious on the long term and targeting of specific MMPs during disease progression will probably be necessary to have a potential therapeutic effect.[643]

2.14 Autophagy

A more recently emerging target is autophagy. Autophagy is an important process for clearing dysfunctional organelles, *e.g.* defective mitochondria, augmenting energy production and preventing tissue damage. Autophagy was shown to be dysfunctional in DMD patient biopsies and *mdx* muscle. A persistent activation of Akt inhibits autophagy via mTOR and its downstream targets, like ribosomal protein S6 and 4E-BP1 (fig. 2.6). mTOR inhibition by itself has been shown to be insufficient to rescue autophagosome formation, indicating also other (pro-autophagy) pathways are involved. In DMD and *mdx* a decreased expression of its downstream targets LC3 II and increase in p62 (LC3 II reduces p62 expression) was observed.[646] pAkt inhibits the pro-autophagy FOXO3 pathway.[647] FOXO3 stimulates autophagy by both increasing LC3 and inhibiting mTOR.[648]

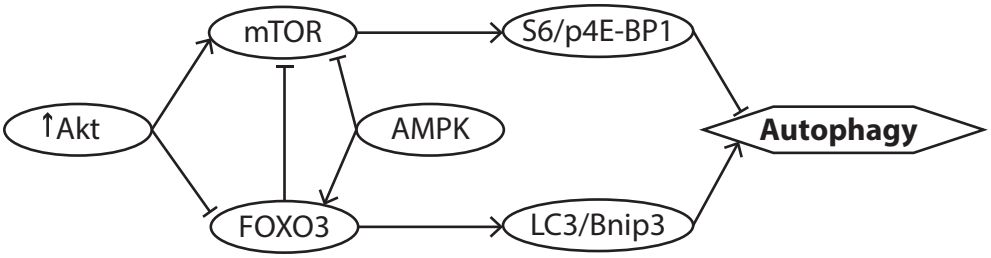

Fig. 2.6: Autophagy
Chronic activation of Akt in Duchenne muscular dystrophy inhibits autophagy by activating the S6/p4E-BP1 pathway through mTOR, which in turn inhibits autophagy, and by blocking FOXO3-mediated LC3/Bnip3 signalling. AMPK can switch on the autophagic pathway by inhibiting mTOR and stimulating FOXO3.

AMP-activated protein kinase (AMPK) is a major metabolic sensor of the energy status inside cells and can switch on the autophagy pathway. The AMPK agonist AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) was a potent trigger of autophagy in *mdx* diaphragm and caused improvement of mitochondrial function, thereby ameliorating histology and function.[649]

In *mdx* mice a long term low-protein diet, a potent autophagy-reactivating treatment, resulted in a decrease in pAkt/mTOR and an increase in LC3 II expression, accompanied by a decrease in p62. This reduced inflammation, fibrosis and myofibre damage and improved functionality.[646]

2.15 Treatment of cardiomyopathy

In most DMD patients cardiomyopathy develops at latter stages of disease. Signs of cardiomyopathy include left ventricular dilation, decreased ejection fraction and increased plasma levels of neuroendocrines. Neuroendocrines (*e.g.* atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and norepinephrine) are cardiac hormones thought to be secreted in response to increasing atrial and ventricular pressure. Cardiomyopathy is nowadays frequently the cause of death, especially since the introduction of assisted ventilation. Therefore treatment of cardiomyopathy is becoming more and more important; however most therapies described above do not or only modestly improve cardiac pathology and function. Several treatments used in cardiomyopathies with other causes, might also be effective in DMD and some of these have been tested in dystrophic models or DMD patients. ACE-inhibitors have already been discussed (see paragraph 2.8). Many patients are nowadays treated with ACE-inhibitors, often combined with β-blockers, when signs of cardiomyopathy start to develop.[565]

In the RA(A)S system (see paragraph 2.8), next to binding to and signalling via the AT receptors, Angiotensin II also stimulates the synthesis of the mineral corticoid aldosterone and emerging data indicate that aldosterone plays an independent role in vascular toxicity and fibrosis. Therefore aldosterone antagonists are widely used and have shown effectiveness in the treatment of cardiomyopathies. Aldosterone binds to aldosterone receptors (*e.g.* mineral corticoid receptors) and sexual hormone receptors. Activation of these mineral corticoid receptors by aldosterone and cortisol plays a role in patients with cardiovascular disease and addition of an aldosterone antagonist to ACE-inhibitor treatment has shown to have an

additional benefit on lowering mortality.[650,651] As described above the competitive antagonist of the aldosterone receptor spironolactone, a diuretic and antihypertensive agent, has been tested in *mdx* mice. However, since this was only in combination with lisinopril, no conclusions can be drawn about the effect of spironolactone itself.[567] Eplerenone, a spironolactone derivative, is a more selective compound, with a higher affinity for mineral corticoid receptors and lower for sexual hormone receptors, thereby reducing the risk of side effects. It has so far only been tested in DMD for treatment of muscle oedema, which is caused by an increase in cytoplasmic Na^+-content and probably contributes to muscle degeneration. In a small study, eplerenone decreased Na^+-concentration in muscles of DMD patients, thereby decreasing oedema and improving muscle strength.[652] Another pilot in one female DMD patient showed the same result of decreasing cytoplasmic sodium and water overload and increasing muscle strength and mobility.[653] A clinical trial to assess the effects of early treatment with eplerenone on cardiomyopathy in DMD patients is currently ongoing (NCT01521546). Eplerenone is thought to have fewer side effects than older aldosterone antagonists, *e.g.* spironolactone and its active metabolite canrenone, due to higher specificity for mineral corticoid receptors. However, a meta-analysis of randomized controlled trials with aldosterone antagonists in systolic heart failure concluded less reduction in mortality for eplerenone compared to spironolactone and canrenone. In addition, it did not have a better side effects profile and is much more expensive.[650]

B-blockers bind to β-adrenergic receptors to inhibit sympathetic effects of binding of (nor)epinephrine to these receptors, thereby reducing the work load on the heart. They are widely used in the treatment of cardiac dysfunction and hypertension. First generation β-adrenoreceptor antagonists are non-selective for β_1- and β_2-adrenergic receptors; whereas second generation β-blockers are relatively selective for β_1-adrenergic receptors, the predominant receptor in the heart.[8] Carvedilol (racemic lipophilic aryloxypropanolamine) causes vasodilatation by non-selective blockage of β-adrenoreceptors and α_1-adrenoreceptors. It has proven therapeutic value as an adjunctive therapy in combination with diuretics and/or ACE-inhibitors in the treatment of various types of heart failure.[654] A small study with carvedilol for six months in four DMD patients with reduced ejection fraction, did not show an improvement compared to controls.[655] In contrast, in a study in 22 muscular dystrophy (DMD or BMD) patients with dilated cardiomyopathy, carvedilol treatment was safe and resulted after six months in a modest improvement in systolic and diastolic functions. Another open trial where 41 patients received carvedilol versus 13 untreated patients, an improved survival rate was observed when solely looked at cardiac failure as cause of death, but not when looked at all-cause mortality. Furthermore no changes in LVEF or BNP plasma levels were observed. In addition, patients with a very low ejection fraction were also treated with an ACE-inhibitor or pimobendan (a PDE3-inhibitor).[656] A randomized, double-blinded, placebo-controlled clinical trial assessing the effect of nebivolol, a β_1-blocker, on the prevention of systolic dysfunction in DMD patients is currently ongoing (NCT01648634). Furthermore two phase IV clinical trials with carvedilol, one with carvedilol (NCT00606775) only and one (NCT00819845) comparing it with the ACE-inhibitor ramipril, should have been performed; however no results are published and their status is unknown.

As described before, combinational treatment with an ACE-inhibitor and a β-blocker is often used for DMD-related cardiomyopathy. A study in 11 symptomatic patients suggested this combination might reduce left ventricular dilation, BNP and ANP levels and may reverse symptoms; however no control group existed.[14] In a study with several types of muscular dystrophy patients, among whom DMD patients, carvedilol plus an ACE-inhibitor improved

left ventricular systolic function, whereas ACE-inhibitors alone did not.[657] A larger retrospective study on DMD patients with heart failure treated with an ACE-inhibitor and β-blocker showed a beneficial effect on long term (over ten years) survival, which was most effective in asymptomatic patients with left ventricular dysfunction.[569] A recent study confirmed this beneficial effect of combinational therapy (ACE-inhibitor and β-blocker) since a significant improvement was seen in ejection fraction compared to before the start of treatment. This was also seen by ACE-inhibitor treatment alone and no additional value of the β-blocker was observed. However, this can be due to the delayed addition of β-blocker therapy.[570]

2.16 Summary

In this chapter various therapeutic strategies that do not aim to restore the expression of the dystrophin protein itself, have been discussed. These vary from genetic approaches to up- or downregulate the expression of other genes to pharmaceutical compounds acting on signalling pathways disturbed in DMD pathogenesis. At the moment the only widely used treatments are corticosteroids and, if signs of cardiomyopathy become apparent, ACE-inhibitors. Unfortunately, while corticosteroids have clear beneficial effects, *i.e.* prolonging ambulation, they also have numerous side effects.

Next to these many compounds have been identified showing positive results in animal models or *in vitro* model systems. Some of these have already been tested in clinical trials, but unfortunately often without very convincing results. One of the problems is that many compounds that seem promising *in vitro* in cells or *in vivo* in animal models often fail when tested in clinical trials or already when tested in (higher) animal models. Partly this is intrinsic to translational research. However, it is to underline the international efforts for more standardization of animal models and outcome measures made in the last years in the frame of activities of the international TREAT-NMD network.[658] This in order to improve predictability of data obtained at pre-clinical stage.[659] In addition, re-evaluation of failed clinical trials is important to design more appropriate pre-clinical testing methods and improve trial protocols in the future. This helps in understanding each other's results and improving model systems.

At the moment, one promising candidate is tadalafil, a PDE5A-inhibitor that is already on the market for other disorders and targets the NO-cGMP signalling pathway to enhance vasodilation. Results in a trial with BMD patients and in a pilot study with DMD patients were promising. Another promising strategy is to target the TGF-β pathway. Hereby, inhibiting both TGF-β and myostatin signalling, *i.e.* by AON-mediated exon skipping of their receptors seems a better strategy than targeting myostatin alone. This has only been tested preclinically, but it could be combined with *DMD* exon skipping to restore both dystrophin expression and enhance muscle formation. However, a concern with myostatin inhibition is to increase the work load on the heart, thereby increasing cardiomyopathy. This does not only account for myostatin inhibition, but for every therapy that mainly improves skeletal muscle function without targeting cardiomyopathy.

A problem will always be that most compounds only target one or a few of the secondary defects, while the underlying cause (lack of dystrophin) is not addressed. Therefore these will only temporarily alleviate the symptoms and should, once available, be combined with a therapy restoring or substituting the dystrophin protein, as is tested for myostatin/TGF-β (receptor) inhibition.