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ABSTRACT

42nd European Muscle Conference: Programme and Abstracts

21–25 Sept 2013, Felix Meritis, Amsterdam

Message from the organisers of the 42nd European Muscle Conference

Dear colleagues,

Bringing back the heart of muscle research to the Netherlands—where in 1682 Antoni van Leeuwenhoek discovered the striation pattern in muscle—we are honoured to host the 42nd annual meeting of the European Society for Muscle Research in the city of Amsterdam.

The **E**uropean **M**uscle **C**onference is hosted by Felix Meritis. A renowned center for the promotion of arts and sciences, Felix Meritis first opened its doors in 1788, and is located on one of Amsterdam's characteristic canals, de Keizersgracht.

The **EMC** is designed to bring together clinicians and researchers with a background in various facets of cardiac, skeletal, and smooth muscle science. Therefore, we have put together an exciting scientific programme that has a strong focus on basic research and its translation to clinical application. Both established and young scientists from all over Europe, the Americas, Asia, and Australia are contributing to the scientific programme. Moreover, exhibitors will show their novel products and will be available for discussions of needs and ideas.

The meeting features poster and platform sessions. The platform sessions highlight emerging topics and provide a platform for young, talented scientists to present their findings.

We look forward to a very exciting and worthwhile 5 days with you, exploring science of mutual interest and setting a framework for future research discussions.

Welcome to Amsterdam!

Coen Ottenheijm, Department of Physiology, ICaR-VU, VU University Medical Center Amsterdam.
Rob Wüst, Department of Physiology, ICaR-VU, VU University Medical Center Amsterdam.
Richard Jaspers, Laboratory for Myology, MOVE Research Institute Amsterdam, VU University Amsterdam.
Jolanda van der Velden, Department of Physiology, ICaR-VU, VU University Medical Center Amsterdam.

Message from the organisers of the Young Investigators Symposium of the 42nd European Muscle Conference

Dear young investigators,

It is a great pleasure to welcome you to the Young Investigators Symposium preceding the **E**uropean **M**uscle **C**onference on Saturday September 21st 2013 in Amsterdam. The **EMC** meeting has a tradition of over 40 years and this year's programme covers the whole muscle field from skeletal muscle to cardiac and smooth muscle.

The 1-day Young Investigators Symposium, which is organised for the first time as an official part of the **EMC**, closely follows the footstep of the **EMC** in terms of research interests. Our target audience are the young participants such as undergraduate and graduate students, but also post-docs and early-career investigators. Obviously, we also welcome more experienced researchers and professors to attend the Symposium to interact, stimulate, challenge and inspire the young investigators.

The aim of our Symposium is to stimulate interaction amongst participants, but also to provide hands-on information on how to become successful in the scientific community. Therefore, we appreciate the contribution of Prof David Eisner as well as the panel committee for their contribution to the symposium. They will be very happy to answer all your career-related questions during the panel discussion, as well as during coffee breaks.

We are thankful that our symposium is supported by a grant from The Physiological Society (United Kingdom) and MEDIA, an FP7-funded European consortium on diastolic heart failure (http://www.diastolicheartfailure.eu).

We are looking forward to seeing you in Amsterdam!

Ilkka Heinonen, Division of Experimental Cardiology, Erasmus University Medical Center Rotterdam.
Josine de Winter, Department of Physiology, ICaR-VU, VUmc, Amsterdam.
Rob Wüst, Department of Physiology, ICaR-VU, VUmc, Amsterdam.

PROGRAMME

Saturday September 21, 2013

YOUNG INVESTIGATORS SYMPOSIUM

13.30 Registration & Coffee/Tea

14.00–14.30 Key Note Lecture by David Eisner—How to succeed in science

This talk is designed to stimulate discussion about some of the issues involved in surviving in science. In many countries science is becoming ever more competitive and less secure. Is science still a good career to embark on? Can one combine science with a normal family life? Can one plan a scientific career or need it be left to chance? How do I select a problem to work on? Do I need a mentor? How to survive rejection? I will illustrate the talk with some personal observations but would strongly recommend everyone to read *Advice to a Young Scientist* by P. B. Medawar (1980).

David Eisner obtained his undergraduate degree in Cambridge and his D. Phil from Oxford. He spent the period 1980–1990 in the Department of Physiology at University College London as a lecturer. From 1990 to 1999 he was Professor of Veterinary Biology at Liverpool University before moving to Manchester as Professor of Cardiac Physiology in October 1999. He is currently the British Heart Foundation (BHF) Professor of Cardiac Physiology.
His research focuses on the regulation of intracellular calcium in the heart and the link between changes of calcium and those of contractility. He is also interested in the relationship between disturbed calcium signalling and arrhythmias. He is currently Editor-in-Chief of the Journal of Molecular and Cellular Cardiology, and President of the Federation of European Physiological Societies.

14.30–15.30 Plenary discussion and Q&A with senior principal investigators

Moderator: Rob Wüst.

Prof David Eisner, Institute of Cardiovascular Sciences, University of Manchester, UK.

Prof Karin R. Sipido, Division of Experimental Cardiology, KU Leuven, Belgium.

Prof Jil C. Tardiff, Department of Cellular and Molecular Medicine, University of Arizona, USA.

Prof Pieter P. de Tombe, Department of Cell and Molecular Physiology, Loyola University Chicago, USA.

15.30–16.00 Coffee break

16.00–17.00 Three 10-min presentations from young investigators
Chairs: Ilkka Heinonen and Josine de Winter.

16.00–16.20 Marion Pauly—*Pharmacological AMPK-activation improves skeletal muscle mitochondrial function and muscle endurance in aged myostatin ko mice.*

16.20–16.40 Johan Lindqvist—*Viral gene delivery restores muscle function in a mouse model of nemaline myopathy.*

16.40–17.00 Anna Müller–*Exercise modifies titin phosphorylation and reduces cardiac myofilament stiffness.*

This symposium is supported by The Physiological Society (United Kingdom) and MEDIA, an FP7-funded European consortium on diastolic heart failure (http://www.diastolicheartfailure.eu):

PROGRAMME

Saturday September 21, 2013

17.00–19.00 **Registration & Drinks & Snacks** (famous 'bitterballen') **for the 'Young & Old' investigators**.

19.00–20.00 **Key Note Lecture**
Dick Swaab—Brain and exercise

Dick Swaab earned his medical and doctoral degrees at the University of Amsterdam, where he became involved in brain research during his third year of medical school. From 1978 until 2005 he has served as director of the Netherlands Institute for Brain Research and since 1979 as Professor of Neurobiology at the medical faculty of his alma mater. He is leader of the research group on Neuropsychiatric Disorders in the Netherlands Institute for Neuroscience. Dick Swaab also holds 3 guest professorships in China, and the USA, is appointed for 2011–2014 Chao Kuang Piu Chair of Zhejiang University, Hangzhou, P.R. China and is "Companion in the Order of the Dutch Lion", bestowed by her Royal Majesty Queen Beatrix of The Netherlands. In 2008 Swaab obtained the Academy medal for his role in national and international neuroscience.
His major research interests focus on brain development, sexual differentiation, aging of the brain, Alzheimer's disease, the neurobiological basis of depression and eating disorders. He has published over 520 papers in SCI journals, authored more than 200 chapters in books, and edited 41 books. Dick Swaab mentored 82 PhD students from which 16 are now full professor. He is author of the 2 volume monograph *The Human Hypothalamus* that appeared in the Handbook of Clinical Neurology series, Elsevier, Amsterdam (1000 pp) and of the Dutch bestseller *We are our Brain*, that is translated in German, Chinese, Taiwanese and Italian, and will appear in 8 more languages. A childrens version of the book (*You are your brain*) has also appeared in Dutch in 2013.
In 1985, Dick Swaab founded the Netherlands Brain Bank (NBB) to serve as a source of clinically and neuropathologically well-documented research tissue. Since its founding, the Brain Bank has provided samples from more than 3,500 autopsies to 500 research groups in 25 countries. He was director of the NBB until 2005.

Sunday September 22, 2013

08.30–08.45 *Coffee/Tea*

08.45–09.00 **Welcome & introduction**

09.00–11.00 **Session I. Mechanotransduction/muscle adaptation**
Chairs: Troy Hornberger and Richard Jaspers

09.00–09.30 Troy Hornberger—*The mechanical activation of mTOR signaling: an emerging role for lysosomal targeting. (I.O1.)*

09.30–10.00 Shin'ichi Takeda—*The molecular mechanism of muscle hypertrophy; roles of nNOS/NO, peroxynitrite and TRPV1.(I.O2.)*

10.00–10.15 A. Judge—*HDAC1 activates foxo in skeletal muscle and is both sufficient and required for skeletal muscle atrophy. (I.O3.)*

10.15–10.30 J. Machado—*Calcitonin gene-related peptide (CGRP) inhibits the autophagy-lysosomal system through cAMP/PKA signaling in isolated skeletal muscles. (I.O4.)*

Sunday September 22, 2013

10.30–10.45 C. Moorwood—*Mechanotransduction* via *the sarcoglycan complex in skeletal muscle. (I.O5.)*

10.45–11.00 R. Piccirillo—*The p97/VCP ATPase is critical in muscle atrophy and for the accelerated degradation of most muscle proteins. (I.O6.)*

11.00–11.30 *Coffee break*

11.30–13.00 **Session II. Muscle regeneration**

Chairs: Fabien Le Grand and Marie-Jose Goumans

11.30–12.00 Fabien Le Grand—*Wnt signaling(s) and skeletal muscle regeneration. (II.O1.)*

12.00–12.30 Marie-Jose Goumans—*Cardiac progenitor cells for heart repair. (II.O2.)*

12.30–12.45 L. Mazelet—*Recovery from skeletal muscle paralysis: studies using embryonic zebrafish. (II.O3.)*

12.45–13.00 R. Mounier—*Ampkα1 regulates macrophage skewing during skeletal muscle regeneration: a link between inflammation and stem cell biology. (II.O4.)*

13.00–15.00 **Lunch break and poster viewing (Sessions I–IV)**

15.00–16.30 **Session III. Cardiac muscle disease—right and left**

Chairs: Walter Paulus and Frances de Man.

15.00–15.30 Walter Paulus—*The failing left ventricle: a novel paradigm for heart failure with preserved left ventricular ejection fraction. (III.O1.)*

15.30–16.00 Frances de Man—*Effect of the pulmonary circulation on the heart. (III.O2.)*

16.00–16.15 V. Sala—*Sustained Met signalling in the heart leads to cardiac compensated hypertrophy and remodelling, which evolve into congestive heart failure. (III.O3.)*

16.15–16.30 F. Suhr—*Beta-parvin knockout in mice causes pathological cardiac adaptations towards physiological volume loading. (III.O4.)*

16.30–17.00 *Coffee break*

17.00–18.30 **Session IV. Excitation–contraction coupling**

Chairs: John Solaro and Pieter de Tombe

17.00–17.30 Karin Sipido—*Electrophysiologic abnormalities in failing cardiac muscle. (IV.O1.)*

17.30–18.00 Pieter de Tombe—*Frank–Starling mechanism. (IV.O2.)*

18.00–18.15 I. Adeniran—*In silico investigation of the electromechanical consequences of the short QT syndrome. (IV.O3.)*

18.15–18.30 M. Reconditi—*Sarcomere-length dependence of the structure of the thick filament studied by X-ray diffraction from intact frog muscle fibres at rest. (IV.O4.)*

18.30–19.30 **Sponsor presentations**

Monday September 23, 2013

08.30–09.00 *Coffee/Tea*

09.00–11.00 **Session V. Large cytoskeletal proteins**

Chairs: Henk Granzier and Coen Ottenheijm

09.00–09.30 Henk Granzier—*Titin and Nebulin: major players in striated muscle function and disease. (V.O1.)*

09.30–10.00 Siegfried Labeit—*Insights from structural biology into novel titin variant alleles implicated in heart failure. (V.O2.)*

10.00–10.15 S. Hunter - *A new network crosslinking thick filaments in the bare region in vertebrate striated muscle – titin kinase links? (V.O3.)*

10.15–10.30 A. Kho—*Titin kinase, maintaining muscle according to demand. (V.O4.)*

10.30–10.45 K. Pelin—*The spectrum of mutations and normal variation in the nebulin gene. (V.O5.)*

10.45–11.00 M. Helmes - *Development of a fast and sensitive (nanoN) force transducer for muscle research. (V.O6.)*

11.00–11.30 *Coffee break*

11.30–13.00 **Session VI. Diaphragm physiology**

Chairs: Leo Heunks and Theodorus Vassilakopoulos

11.30–12.00 Leo Heunks—*The effects of critical illness on respiratory muscle function. (VI.O1.)*

12.00–12.30 Theodorus Vassilakopoulos—*The respiratory muscles exhibit significant adaptability and plasticity. (VI.O2.)*

12.30–12.45 P. Hooijman—*Diaphragm muscle fiber strength is severely reduced in mechanically ventilated ICU patients and is restored by a fast troponin activator. (VI.O3.)*

12.45–13.00 L. Larsson—*Time-course analysis of mechanical ventilation-induced diaphragm contractile muscle dysfunction. (VI.O4.)*

13.00–15.00 **Lunch break and poster viewing (Sessions V–VIII)**

15.00–16.30 **Session VII. Muscular dystrophies and congenital myopathies**

Chairs: Nigel Clarke and Silvere van der Maarel

15.00–15.30 Silvere van der Maarel—*New developments in facioscapulohumeral muscular dystrophy. (VII.O1.)*

15.30–16.00 Nigel Clarke—*Recent developments in the congenital myopathies. (VII.O2.)*

16.00–16.15 W. Eilers—*Trans-splicing aav vectors to deliver full-length dystrophin in mdx mice. (VII.O3.)*

16.15–16.30 C. Latroche—*Alterations of the microvascular system in chronic myopathies and functional repercussions on the muscle tissue. (VII.O4.)*

16.30–17.00 *Coffee break*

17.00–18.30 **Session VIII. Smooth Muscle**

Chairs: Geerten van Nieuw Amerongen and Sarah George

17.00–17.30 Sarah George—*Wnt-signaling in vascular SMCs. (VIII.O1.)*

17.30–18.00 Rama Krishnan—*Contraction of the airway smooth muscle cell in asthma. (VIII.O2.)*

18.00–18.15 I. Heinonen—*Nitric oxide synthase inhibition, with and without inhibition of prostaglandins, reduces blood flow similarly in different human skeletal muscles. (VIII.O3.)*

18.15–18.30 E. Konik—*Vascular reactivity in pulmonary arterial hypertension (PAH): role of myosin light chain phosphatase and nonmuscle myosin. (VIII.O4.)*

18.30–19.00 ***Business Meeting***

Tuesday September 24, 2013

08.30–09.00 *Coffee/Tea*

09.00–10.30 **Point—Counterpoint**

Ca^{2+}-perturbations versus Sarcomere dysfunction in cardiac disease. What matters most? *Moderator*: Karin Sipido. *(PC.O1.)*

09.00–09.30 David Eisner—*Point: Ca^{2+}-perturbations cause cardiac dysfunction*

09.30–10.00 John Solaro—*Counterpoint: sarcomeric dysfunction is the cause of cardiac dysfunction. (PC.O2.)*

10.00–10.30 **Discussion between speakers and audience moderated by Karin Sipido**

10.30–11.00 *Coffee break*

11.00–13.00 **Session IX. Mutations in inherited cardiomyopathies**

Chairs: Tjeerd Germans and Michelle Michels

11.00–11.30 Carolyn Ho—*Sarcomere mutations in hypertrophic cardiomyopathy. (IX.O1.)*

11.30–12.00 Yigal Pinto—*Role of miRNA in inherited cardiomyopathies. (IX.O2.)*

12.00–12.15 J. Montag—*The ß-myosin mutation R453C in Familial Hypertrophic Cardiomyopathy leads to altered crossbridge kinetics. (IX.O3.)*

12.15–12.30 C. Rowlands—*Calcium handling in the ACTC E99 K transgenic mouse model of hypertrophic cardiomyopathy (HCM). (IX.O4.)*

12.30–12.45 K. Spaendonck-Zwarts—*TTN mutations are common in families with peripartum cardiomyopathy and dilated cardiomyopathy. (IX.O5.)*

12.45–13.00 B. Tosi—*E163R cardiomyopathy-related cTnT mutation affects Ca^{2+}-regulation and energetics of cardiac myofilaments in a murine model. (IX.O6.)*

13.00–15.00 **Lunch break and poster viewing (Sessions IX, X, XI and XII)**

15.00–17.00 **Session X. Regulation of cardiac muscle contraction in health and disease**

Chairs: Corrado Poggesi and Diederik Kuster

15.00–15.30 Samantha Harris—*Role of cardiac myosin binding protein C in the heart and effects of haploinsufficiency/phosphorylation. (X.O1.)*

15.30–16.00 Jil Tardiff—*Mutations in thin filament proteins—current state. (IX.O2.)*

16.00–16.15 O. Cazorla—*Urban carbon monoxide pollution aggravates heart failure through oxidative stress. (IX.O3.)*

16.15–16.30 A. Kovacs—*Right ventricular cellular contractile function is altered and differently modified by antiplatelet agents in a rat model of post-ischemic heart failure. (IX.O4.)*

16.30–16.45 D. Miranda-Silva—*Reverse remodelling in Wistar rats after surgical removal of chronic pressure-overload. (IX.O5.)*

16.45–17.00 P. Wijnker—*Phosphorylation of protein kinase C sites SER42/44 blunts enhanced length-dependent activation in response to protein kinase A. (IX.O6.)*

17.30–18.30 **Guided tours to Observatory of Felix Meritis**

19.30 ***Evening Social Event Scheepvaartmuseum (Kattenburgerplein 1)***

19.30 *Drinks and start of guided tour (limited availability)*

20.30 *Diner*

Wednesday September 25, 2013

08.30–09.00 *Coffee/Tea*

09.00–10.45 **Session XI. Muscle physiology: extreme functional demands**

Chairs: Willem van der Laarse and Coert Zuurbier

09.00–09.30 Robert Balaban—*Systems biology of muscle mitochondria. (XI.O1.)*

09.30–10.00 Andrew Murray—*Cardiac and skeletal muscle metabolism in high altitude hypoxia. (XI.O2.)*

10.00–10.15 F. Graça—*Epinephrine restrains the fasting-induced catabolic effects on skeletal muscle protein breakdown. (XI.O3.)*

10.15–10.30 S. Lamon—*Acute regulation of the stars signalling pathway in response to endurance and resistance exercise. (XI.O4.)*

10.30–10.45 D. Salvadego—*Skeletal muscle oxidative function after a 10-day exposure to hypoxia and microgravity. (XI.O5.)*

10.45–11.15 *Coffee break*

11.15–13.00 **Session XII. Mitochondrial function in health and disease**

Chairs: Ger Stienen and Christoph Maack

11.15–11.45 Marcella Canton—*Contribution of mitochondrial ROS formation to myofibrillar protein oxidation in cardiac and muscle disease. (XII.O1.)*

11.45–12.15 Christoph Maack—*Regulation of mitochondrial ROS formation in cardiac myocytes. (XII.O2.)*

12.15–12.30 J. Fauconnier—*TNFα-mediated caspase-8 activation induces ROS production and trpm2 activation in adult ventricular myocytes. (XII.O3.)*

12.30–12.45 E. Fowler—*Reduced expression of creatine kinase in rat failing right ventricle causes diastolic dysfunction in ventricular myocytes. (XII.O4.)*

12.45–13.00 R.C.I. Wüst—*NADH and FAD kinetics reveal altered mitochondrial function in right ventricular heart failure. (XII.O5.)*

13.00 **Awards and Closing Remarks**

Sunday September 22, 2013

Session I. Mechanotransduction/muscle adaptation

#	Page	Presenter	Title
1	64	Baán J.	The *compact* mutation of myostatin causes a glycolytic change in the phenotype of skeletal muscles.

Sunday September 22, 2013

Session I. Mechanotransduction/muscle adaptation

#	Page	Presenter	Title
2	64	Ballak S.B.	Increased mass rather than specific tension causes the increased force generating capacity in hypertrophied adult and old mouse plantaris muscle.
3	65	Barnhill E.	Quantification of limb neuromuscular activation via viscoelastic property mapping with magnetic resonance elastography.
4	65	Bartels E.M.	Non-invasive assessment of muscle function—acoustic myography re-invented.
5	66	Barton E.R.	Masticatory muscles do not undergo atrophy in space.
6	66	Bastide B.	Effects of a 21 day Bed Rest on the PI3/AKT pathway: role of O-GlcNAc
7	67	Desplanches D.	Treatment with trichostatin A attenuates unloaded-induced skeletal muscle atrophy.
8	67	Dijk van M.	Compared to 10 mo old mice, 24 mo old male c57/bl6 mice show characteristics of sarcopenia and anabolic resistance.
9	68	Gondin J.	Muscle damage after neuromuscular electrostimulation assessed by multimodal magnetic resonance imaging.
10	68	Flück M.	Tenascin-C is quantitatively related to angiotensin-modulated muscle growth in man.
11	69	Harzer W.	Gene expression in masseter muscle after orthognathic surgery in comparison with untreated healthy controls.
12	69	Juffer P.	Fluid shear stress stimulates growth factor expression and nitric oxide production by myotubes.
13	70	Kanzaki N.	Effect of glucosamine-containing supplement on knee pain and locomotor functions in subjects with knee pain.
14	70	Kocsis T.	Changes in the tissue glycogen distribution in a hypermuscular mice model.
15	71	Kopylova G.V.	Study of influence of hyperthyroidism on calcium regulation of muscle contraction in slow and fast skeletal muscle of rabbit with an in vitro motility assay.
16	71	Kröss M.	Cross-bridge attachments in fibre types of human skeletal muscle.
17	72	Liu J.	Age-related changes in neurotrophic factors in human extraocular and limb muscles.
18	72	Meijer J.P.	Differences in contractile properties of single muscle fibres from bodybuilders, wrestlers and untrained controls.
19	73	Mirzoev T.M.	The key markers of proteolytic signaling pathways in m. vastus lateralis among alcohol consumers.

Sunday September 22, 2013

Session I. Mechanotransduction/muscle adaptation

#	Page	Presenter	Title
20	73	Mithraratne K.	Mathematical representation of fascicular orientation in skeletal muscles and its implications on passive and active deformations.
21	74	Moro T.	Molecular signalling response to short duration high intensity/low volume resistance training in human skeletal muscle.
22	74	Nemirovskaya C.L.	The effect of calpain inhibition on signaling pathways in m.soleus under hindlimb unloading of rats.
23	75	Reggiani C.	Effects of endurance training, resistance training and neuromuscular electrical stimulation (NMES) on myonuclei and satellite cells density.
24	75	Schöck F.	Alp/enigma family proteins cooperate in Z-disc formation and myofibril assembly.
25	76	Shenkman B.S.	Signaling effects in reloaded muscle: eccentric-like events.
26	76	Silveira W.A.	CAMP/PKA signaling activation induces foxo1 phosphorylation and suppresses ubiquitin–proteasome system in skeletal muscle.
27	77	Stevens L.	Effects of hypo/hypergravity variations on mice soleus plasticity.
28	77	Suhr F.	Divergent in vivo muscle contractions cause distinct skeletal muscle focal adhesion/costamere adaptations and related downstream signaling pathways.
29	78	Zacharewicz E.	Regulation of skeletal muscle micrornas in young and old adults following acute resistance exercise.
30	78	Zong H.	Suppression of mTORC1 activation in acid-α-glucosidase deficient disease is ameliorated by leucine supplementation.

Session II. Muscle regeneration

#	Page	Presenter	Title
1	79	Akpulat U.	Klf5 modulates proliferation and differentiation of myoblasts.
2	79	Berkholz J.	SkNAC: A multi-functional regulator of myogenesis and skeletal muscle regeneration.
3	80	Durcan P.J.	Do humans and flies share the same molecular mechanisms in regulating muscle cell fusion events?
4	80	Flück M.	Calcium/Calmodulin-dependent kinase preserves contractility of injured skeletal muscle.

Session II. Muscle regeneration

#	Page	Presenter	Title
5	81	Gallo S.	HGF receptor agonists protect cardiomyocytes from $CoCl_2$-induced cell injury.
6	81	Gondin J.	Role of macrophagic HIFS in the resolution of inflammation during skeletal muscle regeneration.
7	82	Guha R.	Deep molecular investigation of skeletal muscle: from satellite cell to muscle tissue
8	82	Joshi S	Role of tead family of transcription factors in skeletal muscle differentiation.
9	83	Kocaefe C.	SFRP2 designates skeletal muscle fibrosis in immobilized muscle.
10	83	Jozkowicz A.	Overexpression of hemeoxygenase-1 in murine myoblasts stimulates blood flow recovery after ischemia.
11	84	Naldaiz-Gastesi N.	Murine muscle engineered from dermal precursors: an in vitro model for skeletal muscle generation, degeneration and fatty infiltration.
12	84	Lindström	Pax7 positive/satellite cells in human extraocular muscles.
13	85	Meijden van der K.	Effects of 1,25-dihydroxyvitamin D on proliferation and differentiation of C2C12 myoblasts.
14	85	Myburgh K.H.	Significant myogenin expression is not required to synthesise small muscle fibres after in vivo injury in transgenic mice.
15	86	Pfuhl M.	MS1/stars—a novel cardiac stress response transcription factor?
16	86	Wójtowicz I.	Functional analysis of αB-crystallin's ortholog in muscles development and sarcomers stabilisation in *Drosophila melanogaster*.

Session III. Cardiac muscle disease—right and left

#	Page	Presenter	Title
1	87	Colman M.A.	The role of the pulmonary veins and left atrium in the remodelled human atria during atrial fibrillation.
2	87	Manders E.	Contractile dysfunction of left ventricular cardiomyocytes in patients with pulmonary arterial hypertension.
3	88	Unger A.	Mena/VASP-dependent anchoring of cytoplasmic actin protects against dilated cardiomyopathy.
4	89	Watanabe D.	Changes in myofibrillar Ca^{2+}-sensitivity with low-frequency force depression.
5	89	Zendzian-Piotrowska M.	Sphingolipid profile in the unifarcted ventricular wall of the rat after myocardial infarct.

Session IV. Excitation–contraction coupling

#	Page	Presenter	Title
1	90	Adamek N.	Temperature and 'fatigue' effects on ATP-induced dissociation of fast and slow actomyosin
2	90	Bengtsson E.	Studies of actin conformational states related to myosin binding
3	91	Bullard B.	Similarities in the regulation of insect flight muscle and cardiac muscle of vertebrates
4	91	Campbell K.S.	Dynamic coupling of regulated binding sites and cycling cross-bridges produces activation-dependent mechanical properties in muscle
5	92	Chung C.S.	Low temperature and transmural effects augment variability in measurements of calcium transients and sarcomere length in unloaded left ventricular cardiomyocytes
6	92	Degens H.	The effects of steady lengthening on force of rat soleus fibres: data to test models of cross-bridge function.
7	93	Katnelson L.B.	Effect of mechanical factors on the rhythm disturbances in cardiomyocytes overloaded with calcium: modeling and experimental validation
8	93	Leite F.S.	Force produced by thick filaments isolated from soleus muscles lacking arginyl-trna-protein transferase (Ate1)
9	94	Papadopoulos S.	The cytoplasmic foot of type 1 ryanodine receptor targets junctionally, retrogradely enhances l-type Ca^{2+}-currents and homotetramerizes
10	94	Reconditi M.	Temperature dependence of myosin filament structure in relaxed mammalian muscle.
11	95	Toral-Ojeda I.	New evidence for calcium mishandling in human Calpain 3 deficient myotubes

Monday September 23, 2013

Session V. Large cytoskeletal proteins

#	Page	Presenter	Title
1	96	Anderson B.R.	Determining the molecular mechanisms that link a titin mutation to cardiomyopathy
2	96	Bobylev A.G.	Study on amyloidogenesis of smooth muscule and striated muscule titin in vitro
3	97	Buck D.	Removal of titin's proximal Ig domains results in differential splicing of titin and alters skeletal muscle function
4	97	Chung C.S.	Shortening of the proximal tandem Ig segment of titin leads to diastolic dysfunction
5	98	Franssen C.	Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat cardiometabolic risk model

Monday September 23, 2013

Session V. Large cytoskeletal proteins

#	Page	Presenter	Title
6	98	Kötter S.	Human myocytes are protected from stress-induced stiffening by chaperone binding to titin spring elements.
7	99	Leber Y.	Filamin C is a highly dynamic Z-disc protein immobilized by Xin and Xirp2
8	99	Luther P.K.	Evidence that h-zone width in vertebrate muscle sarcomere is quantised in steps of 43 nm
9	100	Mártonfalvi Zs.	Large-scale molten-globule dynamics contribute to titin contractility
10	100	Mártonfalvi Zs.	Low-force structural transitions in single titin molecules reflect a memory of contractile history
11	101	Marttila M.	Actin and tropomyosins show different binding affinities to mutant nebulin in vitro
12	101	Müller A.E.	Exercise modifies titin phosphorylation and reduces cardiac myofilament stiffness
13	102	Orfanos Z.	Xin repeat proteins interact with SH3 domains of nebulin and nebulette during myofibril formation and remodeling
14	102	Ottenheijm C.A.C.	Deleting exon 55 from the nebulin gene induces severe muscle weakness in a mouse model for nemaline myopathy
15	103	Rudolph F.	Live imaging of titin in sarcomere assembly and disassembly
16	103	Sarparanta J.	Characterization of CAPN3-dependent proteolysis of C-terminal tintin
17	104	Schöck F.	The nebulin family protein *Lasp* regulates thin filament length in myofibrils
18	104	Siebrecht S.	Ultrastructural localisation of titin during sarcomere assembly in regenerating vertebrate skeletal muscle after intoxication with notexin
19	105	Suhr F.	The type of chronic in vivo mechanical loading of rat skeletal muscles induces alterations of extracellular matrix structural constituents

Session VI. Diaphragm physiology

#	Page	Presenter	Title
1	106	Corpeño R.	Sparing of muscle mass and function by passive loading in an experimental intensive care unit model
2	106	Ferreira L.F.	Diaphragm contractile dysfunction in heart failure: role of the $p47^{phox}$ subunit of NADPH oxidase
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Session X. Regulation of cardiac muscle contraction in health and disease

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11	146	Nagy L.	Ca^{2+}-sensitizing effects of OR-2828 in permeabilized cardiomyocytes.
12	147	Nikitina L.V.	Study of effects of tropomyosin isoforms on the regulation of actin–myosin interaction in myocardium with in vitro motility assay.
13	147	Pinali C.	3D reconstruction of subsarcolemmal sarcoplasmic reticulum of sheep cardiac myocytes using serial block face SEM.
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Session X. Regulation of cardiac muscle contraction in health and disease

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INVITED SPEAKERS AND ORAL PRESENTATIONS PER SESSION AND DAY

Sunday 22 September 2013

I. Mechanotransduction/muscle adaptation

Chairs: Troy Hornberger and Richard Jaspers.

I.O1.

The mechanical activation of mTOR signaling: an emerging role for lysosomal targeting

T. A. Hornberger

School of Veterinary Medicine, Department of Comparative Biosciences, University of Wisconsin. Madison, USA

Mechanical stimuli play a major role in the regulation of skeletal muscle mass and the maintenance of muscle mass contributes significantly to disease prevention and issues associated with the quality of life. Although the link between mechanical signals and the regulation of muscle mass has been recognized for decades, the mechanisms involved in converting mechanical information into the molecular events that control this process remain poorly defined. Nevertheless, our knowledge of these mechanisms is advancing. For example, recent studies indicate that signaling through a protein kinase called the mammalian target of rapamycin (mTOR) plays a central role in this event. Hence, this seminar will begin with a summary of the evidence which implicates mTOR in the mechanical regulation of muscle mass. We will then explore the current knowledge of how mechanical stimuli are thought activate mTOR signaling. Specifically, we will focus on the emerging evidence which suggests that the lysosome may be a key site for integrating the molecular signals that ultimately drive the mechanical activation of mTOR signaling.

I.O2.

The molecular mechanism of muscle hypertrophy; roles of nNOS/NO, peroxynitrite and TRPV1

S. Takeda

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Skeletal muscle atrophy occurs in aging and pathological conditions including cancer, diabetes and AIDS. Treatment of atrophy is based on either preventing protein degradation pathways, these are activated during atrophy, or activating protein synthesis pathways, those induce muscle hypertrophy.

Neuronal nitric oxide synthase (nNOS), the enzyme that produces nitric oxide, is mainly localized at the sarcolemma, as a peripheral member of the dystrophin–glycoprotein complex. We previously demonstrated that unloading activated nNOS and promoted muscle atrophy by activating FoxO3a (Suzuki et al. 2007). In addition, we observed that re-growth of atrophied muscle after reloading was also impaired in nNOS-null mice, suggesting that nNOS was indispensable for muscle growth and/or hypertrophy.

Here, we show that the overload-induced hypertrophy was prevented in *nNOS*-null mice. Moreover, nNOS was transiently activated within 3 min after the initiation of overload. This activation promoted formation of peroxynitrite, a reaction product of nitric oxide with superoxide, which was derived from NADPH oxidase 4 (NOX4). Nitric oxide and peroxynitrite then activated TRPV1, resulting in an increase of intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) that subsequently triggered activation of mammalian target of rapamycin (mTOR). Notably, administration of the TRPV1 agonist, capsaicin, induced hypertrophy without overload, and alleviated the unload- or denervation-induced atrophy. These findings identify nitric oxide, peroxynitrite and $[Ca^{2+}]_i$ as the critical mediators that convert a mechanical load into an intracellular signaling pathway, and suggested that TRPV1 could be a novel therapeutic target for treatment of muscle atrophy (Ito et al. 2013).

I.O3.

HDAC1 activates foxo in skeletal muscle and is both sufficient and required for skeletal muscle atrophy

A. W. Beharry[1], P.B. Sandesara[1], B.M. Roberts[1], L.F. Ferreira[2], S.M. Senf[1], A.R. Judge[1]

[1]University of Florida, Department of Physical Therapy, Gainesville, Florida, USA; [2]University of Florida, Department of Applied Physiology and Kinesiology, Gainesville, FL, USA

The Forkhead boxO (FoxO) transcription factors are necessary for the skeletal muscle atrophy associated with several pathophysiological conditions, including muscle disuse, cancer cachexia and sepsis. Therefore, identifying mechanisms which regulate FoxO activation is important from a biological and clinical perspective. Recent data from our lab indicates that FoxO-mediated induction of the skeletal muscle atrophy program is controlled via reversible lysine acetylation. Therefore, the purpose of the current study was to determine whether specific histone deacetylase (HDAC) proteins contribute to FoxO activation and induction of the muscle atrophy program. Through the use of pharmacological inhibitors to block HDAC activity (TSA, MS-275 and MC-1568) we demonstrate that class I and II HDACs contribute to FoxO activation and atrophy gene expression in response to nutrient deprivation in C2C12 skeletal myotubes, and are necessary for muscle fiber atrophy during nutrient deprivation in mice. We further demonstrate that inhibition of class I HDACs, via treatment of mice with MS-275, during 10-days of hind limb cast-immobilization attenuates $\sim$40 % of disuse muscle fiber atrophy and completely abolishes the depression in maximum specific isometric force. Through transfection of whole rat solei with WT HDAC1 or d.n. HDAC1 expression plasmids we further demonstrate that HDAC1 is both sufficient to cause muscle atrophy and is necessary for fiber atrophy associated with muscle disuse. These findings solidify the importance of class I HDACs in the regulation of FoxO and the muscle atrophy program, and indicate that therapeutics targeting class I HDACs are feasible countermeasures to impede muscle atrophy and contractile dysfunction.

I.O4.

Calcitonin gene-related peptide (CGRP) inhibits the autophagy-lysosomal system through cAMP/PKA signaling in isolated skeletal muscles

J. Machado[1], D.A. P. Gonçalves[1], W.A. Silveira[1], L.H. Mandredi[1], L.Z. C. Paula[1], E.A. Filippin[2], N.M. Zanon[1], I. C. Kettelhut[2], L.C. C. Navegantes[1]

[1]Department of Physiology; [2]Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto - University of São Paulo, Brazil

Introduction The present study tested the hypothesis that CGRP, a neuropeptide released by motor neuron, regulates the autophagy-lysosomal proteolytic system in rat skeletal muscles.

Methods *Extensor digitorum longus* EDL muscles from rats ($\sim$80 g) previously denervated (sciatic nerve section by 7d) or sham were isolated and incubated with CGRP, at different concentrations, and the proteolytic activity of ubiquitin–proteasome, Ca^{2+}-dependent and lysosomal systems, as well as gene expression and protein content of different components of these systems were evaluated.

Results and discussion In innervated and denervated muscles, CGRP (10^{-6} M) elevated cAMP levels and activated PKA/CREB signaling. In parallel, CGRP (10^{-6} to 10^{-10} M) reduced the basal and the denervation-induced increase in overall proteolysis, in a dose-dependent manner. The N-Vanillylnonanamide (10^{-4} M), a TRPV1 agonist that leads CGRP release, reduced the overall proteolysis. The anti-proteolytic effect of both CGRP and N-Vanillylnonanamide was abolished by CGRP8-37 (10^{-6} M), a CGRP receptor-antagonist. The anti-proteolytic effect of CGRP was associated with the inhibition of lysosomal activity and decreased mRNA levels of cathepsin L, LC3b, Gabarapl1, Beclin1 and protein content of LC3-II in both innervated and denervated muscles. H89, a PKA antagonist, abolished the inhibitory CGRP effect in the conversion of LC3-I to LC3-II, and Db-cAMP, a cAMP analogue mimetized the anti-proteolytic CGRP effect on overall and lysosomal proteolysis.

Conclusion This study shows that CGRP, through the cAMP/PKA signaling, exerts a direct inhibitory effect on skeletal muscle protein breakdown by suppressing the autophagy/lysosomal proteolysis.

I.O5.

Mechanotransduction via the sarcoglycan complex in skeletal muscle

C. Moorwood, A. Filippou, J. Spinazzola, H. Cho, B. Keyser, E. R. Barton

University of Pennsylvania School of Dental Medicine, Department of Anatomy and Cell Biology, Philadelphia, USA

The sarcoglycans (α, β, γ and δ) are transmembrane glycoproteins that form a subcomplex of the dystrophin glycoprotein complex (DGC) in skeletal muscle, and are mutated in various forms of Limb Girdle Muscular Dystrophy. In response to lengthening contractions of skeletal muscle, γ-sarcoglycan is phosphorylated at tyrosine 6 of the intracellular domain. This phosphorylation event is necessary for normal downstream ERK1/2 signalling, which promotes growth and survival.

We used an in vitro primary myotube stretching assay to investigate further components of the γ-sarcoglycan-dependent mechanotransduction pathway and to isolate the responses to passive loading from

the responses to active contraction. We found that p70S6 kinase (p70S6K) had greater basal activation in γ-sarcoglycan-null than in wild type primary myotube cultures, and that p70S6K activation in response to stretching occurred earlier, but was more transient, in γ-sarcoglycan-null myotubes. Returning to an in vivo model, we found that p70S6K was differentially phosphorylated in γ-sarcoglycan-null muscles, exhibiting an increase in hyper-phosphorylated forms. This may reflect the increased basal activity of ERK1/2 in γ-sarcoglycan-null muscles, since ERK1/2 phosphorylates p70S6K at sites that 'prime' it for activation. This in turn may underlie the more rapid activation of p70S6K in response to stretch in the absence of γ-sarcoglycan in primary myotube cultures.
These findings contribute to our understanding of the role of abnormal load-sensing and growth/survival signalling in the pathogenesis of muscular dystrophies where the sarcoglycan complex is absent.

I.O6.

The p97/VCP ATPase is critical in muscle atrophy and for the accelerated degradation of most muscle proteins

R. Piccirillo[1], A.L. Goldberg[2]

[1]IRCCS - "Mario Negri" Research Institute, Oncology Department, Milan, Italy; [2]Harvard Medical School, Cell Biology Department, Boston, USA

The p97/VCP ATPase complex facilitates the extraction and degradation of ubiquitinated proteins from larger structures. We therefore studied if p97 participates to the rapid degradation of myofibrillar proteins during muscle atrophy. Electroporation of a dominant negative p97 (DNp97), but not the WT, into mouse muscle reduced fiber atrophy caused by denervation and food deprivation. DNp97 (acting as a substrate-trap) became associated with specific myofibrillar proteins and its cofactors, Ufd1 and p47, and caused accumulation of ubiquitinated components of thin and thick filaments, which suggests a role for p97 in extracting ubiquitinated proteins from myofibrils during atrophy. DNp97 expression in myotubes reduced overall proteolysis by proteasomes and lysosomes and blocked the accelerated proteolysis induced by FoxO3, which is essential for atrophy. Expression of p97, Ufd1 and p47 increases following denervation, at times when myofibrils are rapidly degraded. Surprisingly, p97 inhibition, though toxic to most cells, caused rapid growth of myotubes (without enhancing protein synthesis) and hypertrophy of adult muscles. Thus, p97 restrains post-natal muscle growth, and during atrophy, is essential for the accelerated degradation of most muscle proteins.

II. Muscle regeneration

Chairs: Fabien Le Grand and Marie Jose-Goumans.

II.O1.

Wnt signaling(s) and skeletal muscle regeneration

F. Le Grand

Institut Cochin, INSERM U1016, CNRS UMR 8104, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Adult skeletal muscle has a remarkable capacity for regeneration. Since muscle fibers are post-mitotic multinucleated cells, regeneration of the muscle tissue relies on a pool of quiescent muscle stem cells located in a niche around the myofibers: the Satellite Cells (SCs). Upon activation following injury or repeated exercise, SCs leave quiescence to proliferate and then differentiate to form new muscle fibers while a sub-population exit the cell cycle to self-renew and replenish the stem cell niche in the new tissue. In the course of this process, signals from the microenvironment instruct cycling SCs and control myogenesis. We previously demonstrated that numerous Wnt molecules are secreted in the local milieu during regeneration and showed that SC self-renewal is in part controlled by non-canonical Wnt7a/PCP signals sent by the regenerating myofibers. Recently, we identified the secreted molecule Rspo1 as a potentiator of Wnt7a signaling in SC. We observed that Rspo1/Wnt7a signaling drives SC migration and fusion during skeletal muscle regeneration, and that lack of *Rspo1* lead to impaired myofiber repair and tissue hypoplasia following injury. To elucidate the roles of the canonical Wnt pathway in SCs, we generated mice with tamoxifen-inducible and SC-specific *ß-catenin-loxP* (Loss-Of-Function) or *ß-catenin-Ex3-Del-loxP* (Gain-Of-Function) gene disruption. Strikingly, we observed that induction of either *ß-catenin* LOF or GOF mutations in SCs lead to the impairment of skeletal muscle regeneration following injury. Taken together, our results demonstrate that both canonical and non-canonical Wnt pathways are necessary for SC function. We will discuss the potential crosstalks between these two faces of an important signaling.

II.O2.

Cardiac progenitor cells for heart repair

M. J. Th. H. Goumans

Department of Molecular Cell Biologie, Leids Universitair Medisch Centrum, Leiden, The Netherlands

II.O3.

Recovery from skeletal muscle paralysis: studies using embryonic zebrafish

L. Mazelet[1], M.O. Parker[2], C.H. Brennan[2], N. Huq[1], R. Ashworth[1]

[1]School of Medicine and Dentistry; [2]School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom

Myofibril organisation is crucial for structure and function of mature skeletal muscle. The pathways that initiate myofibril assembly, organisation and growth during development remain undefined. Studies in zebrafish embryos have shown that paralysis causes skeletal muscle myofibril disruption. In this study we have tested whether contraction drives myofibril organisation in developing muscle fibres. The effect of paralysis and subsequent recovery on myofibril bundling in fast and slow muscle and swimming behaviour was examined. Movement was inhibited by incubation of embryos in anaesthetic (Tricaine methanesulfonate 0.03 % w/v) for 7 h from the initiation of movement [17–24 h postfertilisation (hpf)]. Embryos were then fixed at 24hpf or allowed to recover movement before fixation at 42hpf. Skeletal muscle structure was examined using whole-mount immunocytochemistry and confocal microscopy. Paralysis caused a significant decrease in myofibril bundle width at 24hpf (4.3 ± 0.2 μm, $n = 8$ in controls compared to 1.9 ± 0.1 μm, $n = 9$ in treated embryos) with partial but not complete recovery at 42hpf (5.7 ± 0.3 μm, $n = 5$ in controls compared to 3.7 ± 0.2 μm, $n = 6$ in treated embryos). Swimming behaviour, assessed by rotations and velocity, was recorded at 1 month post fertilisation (pf) using EthovisionTM. Results showed no differences in baseline swimming or velocity in treated compared to control fish. Further tests will establish the time course of recovery. The zebrafish embryo provides an

accessible, high throughput model to study contraction-driven signalling and myofibril development in vivo.

III. Cardiac muscle disease—right and left

Chairs: Walter Paulus and Frances de Man.

III.O1.

The failing left ventricle: a novel paradigm for heart failure with preserved left ventricular ejection fraction

W. J. Paulus

Department of Physiology, VU University Medical Center, Amsterdam, Institute for Cardiovascular Research, The Netherlands

Myocardial structure, cardiomyocyte function and intramyocardial signaling were recently shown to be specifically altered in heart failure with preserved left ventricular (LV) ejection fraction (HFPEF). Based on these findings, a new paradigm for LV remodeling in HFPEF is proposed. This paradigm identifies coronary microvascular inflammation resulting from a systemic proinflammatory state induced by comorbidities as the cause of myocardial structural and functional alterations. Based on this paradigm, LV remodeling in HFPEF differs from LV remodeling in heart failure with reduced LV ejection fraction (HFREF), in which LV remodeling is driven by loss of cardiomyocytes because of accelerated autophagy, apoptosis and necrosis.

The new paradigm presumes the following sequence of events in HFPEF: (1) A high prevalence of comorbidities such as overweight/obesity, diabetes mellitus, chronic obstructive pulmonary disease and salt sensitive hypertension induce a systemic proinflammatory state; (2) A systemic proinflammatory state causes coronary microvascular endothelial inflammation; (3) Coronary microvascular endothelial inflammation reduces nitric oxide (NO) bioavailability, cyclic guanosine monophosphate (cGMP) content and protein kinase G (PKG) activity in adjacent cardiomyocytes; (4) Low PKG activity favours hypertrophy development and raises resting tension because of hypophosphorylation of titin; (5) Both stiff cardiomyocytes and interstitial fibrosis contribute to high diastolic left ventricular (LV) stiffness and HF development.

The new HFPEF paradigm shifts emphasis from left ventricular (LV) afterload excess to coronary microvascular inflammation. This shift is supported by a favourable Laplace relationship in concentric LV hypertrophy and by all cardiac chambers showing similar remodeling and dysfunction.

Future therapeutic strategies in heart failure should take into account the different mechanisms that drive LV remodeling in HFPEF and HFREF.

III.O2.

Effect of the pulmonary circulation on the heart

F. S. de Man

Department of Pulmonology, VU University Medical Center, Institute for Cardiovascular Research, Amsterdam, The Netherlands

In contrast to the left ventricle, the right ventricle is a thin-walled and highly compliant pump-system, which tolerates pressure-loading conditions poorly. In pulmonary arterial hypertension (PAH) however, the right ventricle has to cope with an almost fourfold increase in pressure due to excessive remodeling of the pulmonary arterioles. How the right ventricle responds to this extreme pressure overload will be subject of my presentation.

By pressure–volume analyses, we determined end-systolic and diastolic elastance to investigate right ventricular (RV) contractility and diastolic function in PAH-patients (NYHA II–III). In addition, we obtained RV tissue samples of PAH-patients (NYHA IV) and donors to assess in more detail morphological, functional and molecular changes of right ventricular cardiomyocytes of PAH-patients. Finally, we also analyzed left ventricular cardiomyocytes of the same patients to investigate whether changes in RV function were induced by systemic factors or a consequence of changes in loading conditions.

In my presentation, I will demonstrate that patients with PAH have increased RV contractility and diastolic stiffness. We observed significant correlations between increased diastolic stiffness and disease severity. Alterations in the extracellular matrix, reduced PKA-mediated phosphorylation and sarcomeric stiffening contributed to increased RV diastolic stiffness in PAH-patients. Different changes in left ventricular cardiomyocyte function were observed, suggesting that changes in loading conditions rather than systemic factors may be an important trigger of changes in the heart of PAH-patients.

III.O3.

Sustained Met signalling in the heart leads to cardiac compensated hypertrophy and remodelling, which evolves into congestive heart failure

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The Hepatocyte Growth Factor (HGF) and its tyrosine-kinase receptor c-Met are involved in many physiological and pathological processes, including heart development and tissue repair after damage. In this work we investigated whether a sustained activation of Met signalling in the heart could influence postnatal cardiac growth.

To this purpose, we generated a transgenic mouse model with cardiac-specific and tetracycline-suppressible expression of Tpr-Met, the oncogenic form of Met receptor. When inducing the Tpr-Met transgene starting from postnatal life, we obtained a model of reversible concentric hypertrophy, with diastolic dysfunction, but preserved systolic properties. Measurement of the levels of SERCA2a and contractile proteins indicated that impaired relaxation and increased stiffness were the underlying basis of diastolic dysfunction. Remodelling of cytoskeleton and extracellular matrix were induced as compensatory mechanisms aimed at preserving heart contractility.

At later ages, the enhanced cardiac growth induced by Tpr-Met evolved into a congestive heart failure, with signs of dyspnaea, ascites, lung oedema and cachexia. The early suppression of Tpr-Met prevented the lethal outcome.

Our results demonstrate that excessive activation of Met signalling can be implicated in the pathogenesis and progression towards heart failure.

IV. Excitation–contraction coupling

Chairs: John Solaro and Pieter de Tombe.

IV.O1.

Electrophysiologic abnormalities in failing cardiac muscle

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IV.O2.

Frank–Starling mechanism

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Myofilament Length Dependent Activation (LDA) underlies the Frank–Starling Law of the heart. The molecular mechanisms that regulate LDA are incompletely understood. We have recently identified troponin-I, and in particular cTnI-Thr144, playing a pivotal role in LDA. Moreover, we found that LDA develops within 5 ms, excluding post-translational modifications as mechanism for LDA. Further, our 2D X-ray diffraction studies indicate that stretch is associated with structural changes in both myosin and troponin in relaxed cardiac muscle. Preliminary studies employing fluorescent probes in skinned isolated myocardium corroborate these data. Finally, expression of a giant isoform of titin in a mutant rat strain is associated with a blunted Frank–Starling response, blunted LDA, and absence of structural rearrangements in troponin and myosin upon stretch in relaxed cardiac muscle. We propose that myofilament length dependent activation is due to the direct transmission prior to activation of titin strain to troponin or weakly-bound cross-bridges inducing structural rearrangements that lead to increase myofilament calcium sensitivity.

IV.O3.

In silico investigation of the electromechanical consequences of the short QT syndrome

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Introduction Short QT Syndrome (SQTS) is associated with accelerated ventricular repolarization, arrhythmias and sudden death. Studies suggest that transmural consequences of accelerated repolarization lead to a substrate favourable to re-entry. Electromechanical consequences of SQTS are less well understood. Therefore, this study employed electromechanical human ventricular models to explore its electromechanical consequences.

Methods Ventricular cell models were produced by coupling the Rice et al. (2008) mechanics model with the ten Tusscher et al. (2006) electrical model for human ventricular cells, incorporating formulations of SQT variants 1–3. Functional effects of the SQTS mutations on Ca^{2+}-transients, sarcomere length shortening and contractile force were evaluated with and without stretch-activated channel current (I_{SAC}). Single cell models were incorporated into multidimensional models of the human ventricle.

Results Without I_{SAC}, the SQTS mutations produced dramatic reductions in the Ca^{2+}-transient amplitude, sarcomere length shortening and contractile force. With I_{SAC}, there was considerable attenuation of these effects. In tissue models, the timing of maximum deformation was delayed in the SQTS setting compared to control.

Conclusion Our simulation qualitatively matches experimental data on the dissociation between ventricular repolarization and the end of mechanical systole in SQT patients. It also suggests that I_{SAC} modulates the functional effects of SQT 1–3 on ventricular mechanical dynamics, raising a question as to whether compensatory changes occur in the SQTS that offset a negative inotropic effect of action potential abbreviation that might occur.

IV.O4.

Sarcomere-length dependence of the structure of the thick filament studied by X-ray diffraction from intact frog muscle fibres at rest

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X-ray patterns were recorded at the ESRF source from bundles of 2–3 fibres of *Rana esculenta* at 4 °C at rest at sarcomere length (SL) 2.0–3.6 μm, and corrected for different diffracting mass with SL and between fibres. The axial pattern was independent of SL in the range 2.0–2.6 μm. For SL 2.6–3.0 μm the intensity and fine structure of the meridional M3 reflection from the axial repeat of the myosin heads was constant, while the intensities of the first myosin layer line (ML1), generated by the helical order of myosin heads, of the 44 nm meridional reflection (M1), associated with myosin-binding protein C (MyBPC), and of the M2 and M5 forbidden reflections (generated by triplet perturbation of the periodicity of the myosin helix), decreased. In the same range the spacing of the M3 reflection increased by ~0.3 %, while those of the forbidden reflections increased by 0.6–0.8 %. The SL dependence of ML1, M1 and of the forbidden reflections indicates that the helical order of the myosin heads and the triplet perturbation depend on the overlap between the thick filament region containing MyBPC and the actin filament. Withdrawing the MyBPC region from overlap suppresses the triplet perturbation and is accompanied by an extension of the thick filament, but does not affect the axial mass projection of individual myosin heads. This suggests that the structure of the thick filament at rest is under the control of "resting links" between MyBPC and actin.

Monday 23 September 2013

V. Large cytoskeletal proteins

Chairs: Henk Granzier and Coen Ottenheijm.

V.O1.

Titin and Nebulin: major players in striated muscle function and disease

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The sliding filament model of the sarcomere, consisting of thin and thick filaments, has been successful in explaining many, but not all, features of striated muscle. Work during the 1980s revealed the existence of two additional filaments: the giant filamentous proteins titin and nebulin. Here I will highlight some of the major discoveries in titin and nebulin fields. Titin is a key determinant of sarcomeric passive stiffness. Titin's stiffness is modulated by isoform variation and phosphorylation by protein kinases. Studies in patients with dilated cardiomyopathy revealed shifts toward more compliant isoforms, an adaptation that offsets increases in passive stiffness based in the extracellular matrix. In contrast, hypophosphorylation of PKA/PKG sites contributes to an increase in cardiomyocyte resting tension in HFpEF. More recently, titin mutations have been recognized as a common etiology of inherited DCM and to cause arrhythmogenic right ventricular dysplasia. Nebulin's role in muscle remained long

nebulous. Recent advances reveal that nebulin plays an important role in the regulation of thin filament length, most likely by stabilizing F-actin assemblies. Recent studies also indicate that nebulin is part of a protein complex that mechanically links adjacent myofibrils. In addition to these structural roles in support of myofibrillar force generation, nebulin has also been shown to regulate directly muscle contraction at the level of the crossbridge. Thus, these recent data all point to nebulin being important for muscle force optimization. Consequently, muscle weakness develops in patients with nemaline myopathy that have mutations in the nebulin gene.

V.O2.

Insights from structural biology into novel titin variant alleles implicated in heart failure

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Mutations in the giant filamentous protein titin (TTN) have been identified to represent one of the more common causes of hereditary cardiomyopathies. The mutations identified include in addition to stop codons (implicated in particular in DCM heart failure), and mis-sense mutations, leading to DCM, HCM and to ARVC-type heart failure types. Diagnostically, there is a need to predict which alleles are disease causing or not, and to which patho-mechanisms they may relate. To date, the mechanisms linking together titin mutations to these different heart failure pathologies are poorly understood. Here, we present structural biology studies on the characterisation of different titin alleles implicated in heart diseases. For this, we compared selected wildtype and mutant titin fragments carrying alleles previously implicated in heart disease by X-ray crystallography, NMR, and SAXCs. Our data suggest that typing of titin domain fold stabilities and shape by NMR and SAXCs could be a useful approach to predict if specific titin alleles are disease-associated: The studied mutations had a significant effect on the stability of the affected titin domains. In addition, they tended also to affect titin interdomain interactions. Our studies suggest that structural biology could be a useful diagnostic titin typing in disease approach and that predictions from in vitro structural biology based approaches can be effectively tested next in higher complex systems, including live myocytes.

V.O3.

A new network crosslinking thick filaments in the bare region in vertebrate striated muscle—titin kinase links?

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Transverse sections of vertebrate striated muscle in the central part of the sarcomere show three distinct features starting from the centre of the M-band: the M-band in which M-bridges link adjacent myosin filaments, the bare-region in which thick filaments have narrow triangular profiles, and the start of the cross-bridge region in which thick filaments have a nearly circular profile with irregular edges. Just before the cross-bridge region the myosin filaments have Y-shaped projections. We have examined this region by electron microscopy and tomography of thin transverse sections of a well-preserved sample of frog skeletal muscle. We have found that this region comprises a distinct network crosslinking adjacent thick filaments. We call this structure the boundary network and the links boundary bridges. Averaging in 3D of thick filaments in this region reveals that the boundary bridges comprise a $\sim$100 Å rod density placed diagonally on the surface of the thick filament. On the M-band side of the density there is a fine link of variable path to an adjacent filament. Titin kinase is a large domain ($\sim$300 residues, $\sim 50 \times 50 \times 35$ Å) at the C-terminus of titin that is known to be located near the M-band. There has been intense research to characterise it and understand its role as a potential force sensor. We are investigating whether the density we observe in the boundary bridges may be partly due to titin kinase.

V.O4.

Titin kinase, maintaining muscle according to demand

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The sarcomere is the basic unit of the contractile machinery of muscle. The correct functioning of muscle relies on the precise arrangement of proteins within the sarcomere. The giant sarcomeric muscle protein titin has an essential role as a molecular ruler in determining sarcomere length. Furthermore, titin has multiple signalling domains, including a C-terminal kinase domain (TK) located in the M-band. This kinase shows unusual sequence features, raising the question whether its main function is catalytic, that of a scaffold, or both. Force spectroscopy experiments and molecular dynamics simulations showed titin kinase could be activated by physiological forces pulling the autoinhibitory domain out of the active site, suggesting a role for the kinase in mechanosensing. Lange et al. reported a signalling complex where open TK interacts with the autophagy adaptor protein nbr1, a complex implicated in load-dependent muscle protein turnover regulation. However, the functional role of TK activity has yet to be demonstrated.

We therefore generated a knock-in mouse line carrying an enzymatically inactive version of titin kinase, where the catalytic aspartate crucial for phosphotransfer in all kinases was mutated to alanine. These transgenic mice live up to 18 months and breed according to normal Mendelian inheritance. Hearts and skeletal muscle from homozygous animals under baseline conditions were analysed for changes in telethonin as well as proteins of the nbr1 signalosome, and related autophagy related proteins. Muscle atrophy was investigated by subjecting homozygous mice to 14-day sciatic denervation.

Our first results suggest that titin kinase is dispensable for cardiac and skeletal muscle development. Telethonin protein as well as phosphorylation levels also appeared normal. However, significant changes in skeletal muscle fibre sizes suggest a non-redundant role in normal muscle maintenance. In addition, TK inactivation affected the regulation of muscle atrophy, in particular the AKT- mTOR growth/repair signalling axis.

In conclusion, although titin kinase activity is not critical or redundant for cardiac and skeletal muscle development, it may play a role in muscle growth and repair during normal maintenance as well as under conditions of atrophy.

V.O5.

The spectrum of mutations and normal variation in the nebulin gene

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Mutations in the nebulin gene (*NEB*) are known to cause nemaline myopathy, distal myopathy and core-rod myopathy, all inherited in an autosomal recessive fashion. To date, we have identified both disease-causing *NEB* mutations in 96 families with nemaline myopathy, in four families with distal myopathy with no nemaline rods, in two families with a distal form of nemaline myopathy and in two families with core-rod myopathy. The mutations are spread all across the 183 exons of the 249 kb *NEB* gene.

The Exome Variant Server (http://evs.gs.washington.edu/EVS/), a collection of exome-sequencing data of 6,503 DNA samples from healthy individuals of African American and European American origin, contains 809 variants in the coding region and conserved splice sites of *NEB* (January 2013). Of the 809 variants, 350 represent known SNPs or novel synonymous changes, 435 variants are novel missense mutations, 12 frameshift mutations, six in-frame deletions, three intronic splice site mutations, two nonsense mutations, and one in-frame insertion. Of the 435 missense mutations 27 are in conserved actin-binding sites of nebulin, whereas seven are in putative tropomyosin-binding sites. Of the approximately 6,000 individuals successfully exome sequenced, 76 were heterozygous carriers of pathogenic *NEB* mutations (including the putative tropomyosin-binding site mutations), which equals a carrier frequency of approximately 1/79, and a disease incidence of approximately 1 in 25,000 for autosomal recessive myopathies caused by *NEB* mutations. Excluding the putative tropomyosin-binding site mutations from the calculations gives a carrier frequency of approximately 1/105 and a disease incidence of approximately 1 in 44,100. The incidence of nemaline myopathy has previously been estimated as 1 in 50,000 (0.02 per 1,000 live births).

In conclusion, normal variation in the giant *NEB* gene is common and the interpretation of mutation analysis results can be difficult. The EVS data show that pathogenic *NEB* mutations are rare among all coding variants ($\sim$6 %), which indicate that nebulin tolerates substantial changes in the amino acid sequence, providing an explanation as to why mutations in such a large gene result in rare disorders.

V.O6.

Development of a fast and sensitive (nanoN) force transducer for muscle research

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A recent exciting development in cardiac research is the ability to do mechanical experiments on intact isolated myocytes. Compared to experiments with isolated permeabilized myocytes, which have been used for many years, the forces measured are lower while the kinetics are faster. Our goal is to be able to control the force development of the myocyte so we can mimic in vivo work loops. To be able to use feedback algorithms for force control, we need a force signal with a high signal-to-noise ratio and high response rate relative to the forces produced by intact cardiac myocytes. To this end we developed a new type of force transducer. A cantilever with a spring constant of approximately 10 Nm is micro machined from a small glass ferrule and interrogated using an interferometer at a frequency of 20 kHz. The resonance frequency of the force transducer in air is >5 and 3 kHz when submerged in water. With such a high resonance frequency the transducer picks up relatively little environmental noise, resulting in a RMS of 6 nN for a typical probe. The resolution that can realistically be achieved with the current design is approximately 10–15 nN. As the probe can be fully submerged in water, variations in solution level have no effect on the force measurement, and the sample can be continuously perfused while measuring force. These characteristics allow the measurement of force transients from intact isolated cardiac myocytes at 37 °C with unprecedented spatial and temporal resolution.

VI. Diaphragm physiology

Chairs: Leo Heunks and Theodorus Vassilakopoulos.

VI.O1.

The effects of critical illness on respiratory muscle function

L. Heunks

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Mechanical ventilation provides life saving support for patients with acute respiratory failure. In these patients mechanical ventilation unloads the respiratory muscles and improves oxygenation. However, recent studies provide evidence that mechanical ventilation comes with adverse effects including ventilator-induced diaphragm dysfunction. Respiratory muscle dysfunction in ventilated patients is of clinical importance as it is associated with increased morbidity, including prolonged weaning from mechanical ventilation.

The respiratory muscles appear more sensitive to the adverse effects of unloading than peripheral skeletal muscles. Studies in animals and in human patients have shown that even brief periods of unloading are associated with diaphragm muscle fiber atrophy and dysfunction. Besides unloading, asynchrony between the ventilator and the patient's neural drive may induce muscle injury.

The pathophysiology of respiratory muscle dysfunction in critically ill patients is incompletely understood, but increased proteolysis, autophagy and impaired muscle protein synthesis are at play. Improved understanding of respiratory muscle dysfunction in the critically ill will help to develop interventions that prevent or treat muscle dysfunction in these patients.

In this presentation, I will discuss the effects of critical illness on respiratory muscle function, the underlying pathophysiology and the clinical importance of respiratory muscle dysfunction in mechanically ventilated patients.

VI.O2.

The respiratory muscles exhibit significant adaptability and plasticity

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When they have to face increased load such as during resistive breathing they contract strenuously. The strenuous respiratory muscle

contractions result in oxidative stress development. At the same time the strenuous contractions result in upregulation of various cytokines within the respiratory muscles. Oxidative stress is a stimulus for the cytokine upregulation since antioxidants attenuate the resistive breathing induced cytokine upregulation. In contrast, nitric oxide is downregulated during resistive breathing within the respiratory muscles. Nitric oxide actively suppresses the resistive breathing induced cytokine upregulation.

When the respiratory muscles are put on rest, such as during controlled mechanical ventilation, they also develop oxidative but not nitrosative stress. This along with calpain and caspase activation lead to proteasome activation and increased proteolysis. This sequence of events culminates in time dependent atrophy of the respiratory muscles. Furthermore, ultrastructural injury develops. The force generating capacity of the respiratory muscles is severely compromised. All the above has been termed ventilator-induced diaphragmatic dysfunction.

VI.O3.

Diaphragm muscle fiber strength is severely reduced in mechanically ventilated ICU patients and is restored by a fast troponin activator

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Importance The pathophysiology of weaning failure in the ICU is complex, and accumulating evidence suggests that diaphragm weakness might play an important role. However, whether true contractile weakness of diaphragm muscle fibers occurs in mechanically ventilated (MV) ICU patients remains unknown.

Methods Diaphragm biopsy specimens were obtained from MV ICU patients ($n = 7$) undergoing laparotomy or thoracotomy (ICU-group) and from patients undergoing elective lung surgery (control-group, $n = 7$). We determined diaphragm muscle fiber cross sectional area (CSA) in cryosections, and in isolated permeabilized diaphragm muscle fibers we determined contractile strength and the ability of the novel fast troponin activator CK-2066260 to improve contractile strength.

Results Our data show that, compared to controls, diaphragm fibers from ICU patients have 29 % smaller ($p = 0.012$) slow-twitch and 37 % smaller fast-twitch ($p < 0.001$) fiber CSA, maximal force generating capacity in ICU-diaphragm fibers is 61 % lower in both slow-twitch ($p < 0.001$) and fast-twitch ($p < 0.001$) fibertypes, pCa_{50} did not differ ($p = 0.23$) in slow-twitch fibers, but was lower in fast-twitch fibers ($p = 0.013$) of ICU patients. The fast troponin activator CK-2066260 (5 μM) improved the force response to Ca^{2+} so that at physiological [Ca^{2+}] the force generation in diaphragm fibers from ICU patients was restored to levels observed in untreated control fibers.

Conclusions This study reveals that diaphragm muscle fibers of MV ICU patients (1) are severely atrophied and weakened, and (2) regain normal strength when exposed to CK-2066260. Thus, fast troponin activators may offer an appealing and novel therapeutic approach to improve diaphragm muscle strength in ICU patients and to facilitate weaning from mechanical ventilation.

VI.O4.

Time-course analysis of mechanical ventilation-induced diaphragm contractile muscle dysfunction

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Controlled mechanical ventilation (CMV) has been suggested to be a key factor triggering the impairment in diaphragm muscle function and the concomitant delayed weaning from the respirator in critically ill intensive care unit (ICU) patients. To date, experimental and clinical studies have primarily focused on early effects on the diaphragm by CMV, or at specific time points. To improve our understanding of the mechanisms underlying the impaired diaphragm muscle function in response to CMV, we have performed time-resolved analyses using an experimental rat ICU model allowing detailed studies of the diaphragm in response to both short- and long-term CMV. Gene and protein expression, protein modifications, protein–protein interactions, muscle fiber size, and regulation of muscle contraction at the single muscle fiber level were studied in the diaphragm muscle in animals exposed to CMV for durations varying between 6 h and 14 days. A decline in maximum force normalized to muscle fiber cross-sectional area (specific force) was observed early (0.25–4 days) and preceded the decline in muscle fiber size observed first after CMV for 5 days and longer. In contrast to observations in limb muscles, the dramatic loss in specific force with a residual capacity around 15 % after 14 days CMV cannot solely be explained by a loss of thick filament proteins and the early onset of oxidative stress and protein modifications of myosin play a dominant role. Specific pharmacological intervention studies are presently undertaken to reduce these effects and preliminary results indicate significant positive effects on diaphragm function.

VII. Muscular dystrophies and congenital myopathies

Chairs: Nigel Clarke and Silvere van der Maarel.

VII.O1.

New developments in facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is associated with an opening of the D4Z4 repeat chromatin structure on chromosome 4

leading to expression of the D4Z4-encoded *DUX4* gene in skeletal muscle. DUX4 is a germline transcription factor that is normally suppressed in somatic tissue.

In the common form FSHD1, somatic expression of *DUX4* is caused by contraction of the D4Z4 array to a size of 1–10 units and is inherited as a dominant trait. In the rare form FSHD2, D4Z4 chromatin opening occurs on normal sized arrays (11–100 units) of both chromosomes 4 because of mutations in the chromatin modifier *SMCHD1* on chromosome 18. SMCHD1 can also act as a modifier of disease severity in FSHD1 families as mutations in *SMCHD1* aggravate disease severity in FSHD1 families with borderline D4Z4 repeat sizes.

SMCHD1 is a chromatin modifier involved in establishment and/or maintenance of CpG methylation and binds directly to the D4Z4 repeat. In FSHD2 patients there is reduced binding of SMCHD1 to D4Z4 and knock down of SMCHD1 in normal myoblasts containing a permissive chromosome 4 leads to the activation of *DUX4*.

The mechanism of repeat-mediated repression of *DUX4* in somatic cells is evolutionary conserved and can be recapitulated in transgenic mice. Mice with normal-sized repeats do not express *DUX4* in skeletal muscle while mice with FSHD-sized repeats show similar epigenetic and transcriptional features of D4Z4 as observed in patient alleles. These mice do not show an overt muscle phenotype and studies are on-going to understand the consequences of ectopic *DUX4* expression in skeletal muscle.

VII.O2.

Recent developments in the congenital myopathies

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Recent advances have impacted the field of congenital myopathies in several ways. The high-throughput sequencing revolution is accelerating the discovery of new disease genes with KLHL40, STIM1, MEGF10 and CCDC78 recently described as causes of various congenital myopathies. The sequencing of broad gene panels or whole exomes is also expanding the phenotypes associated with well-established disease genes. New phenotypes associated with mutations in large disease genes such as TTN, RYR1 and MYH7 have emerged and new clinical patterns have even been described for ACTA1 and TPM2. There has also been progress in understanding disease pathogenesis and in developing treatments, although more slowly. Several reports suggest that patients with congenital myopathies can respond to cholinesterase inhibitors such as pyridostigmine, implicating abnormal neuromuscular transmission in the pathogenesis of muscle dysfunction. Troponin activators continue to show promise in addressing the abnormal sarcomeric dynamics present in many congenital myopathies. Despite these advances, the basis of muscle weakness in many congenital myopathies remains uncertain, and the causes of many phenotypic aspects such as low muscle bulk and selective muscle weakness remain elusive. Hint abound that there is much still to learn about the normal function of skeletal muscle and the ways that force generation can be perturbed by genetic mutations.

VII.O3.

Trans-splicing AAV vectors to deliver full-length dystrophin in mdx mice

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Duchenne muscular dystrophy is an X-linked recessive muscle disease which affects 1/4,000 male births and is caused by the absence of dystrophin. Dystrophin is a 427 kDa protein which links the actin cytoskeleton to the extracellular matrix and its absence in skeletal and cardiac muscle causes progressive muscle degeneration which leads to premature death around age 25.

Restoring body-wide expression of full-length dystrophin presents a major challenge because of its $\sim$11.5 kb coding sequence. Adeno-associated virus (AAV) is capable of systemic gene delivery, but its packaging capacity of only $\sim$4.7 kb has limited its use to the delivery of truncated dystrophin molecules. However, these may not fully restore skeletal and heart muscle function.

We therefore aimed to restore full-length dystrophin expression in skeletal muscle of dystrophic mdx mice by trans-splicing of three separate constructs delivered by AAV (TT-AAV), each encoding $\sim$33 % of GFP-labelled dystrophin. 4 Month-old (mature) and 7 day-old (neonatal) mdx males received an injection into *m. tibialis anterior* of 5×10^{10} vg and 1×10^{10} vg, respectively. Muscles were harvested after 7 months (mature) and 6 weeks (neonatal). We detected 6 (mature; range 2–8) and 8 (neonatal; range 2–21) fibres with sarcolemma-localised GFP expression in TT-AAV-injected muscles, indicating correct assembly of the full-length dystrophin coding sequence. Furthermore, we detected re-localisation of the dystrophin-associated protein β-dystroglycan to the sarcolemma.

These findings demonstrate the feasibility of restoring full-length dystrophin expression using AAV-vectors, which may provide great clinical benefit to patients after optimisation of the TT-AAV system to increase dystrophin expression levels.

VII.O4.

Alterations of the microvascular system in chronic myopathies and functional repercussions on the muscle tissue

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Duchenne Muscular Dystrophy (DMD), the most frequent neuromuscular disorder in school-age children, is a progressive disease characterised by an absence of dystrophin, affecting about 1:3,500 live male birth and inevitably leading to death. Numerous studies focused on muscle lesions observed in this disease, especially myofiber necrosis/regeneration, endo- and perimysial fibrosis, and adipocyte infiltration. In a context of dystrophinopathy, our aim was to decipher alterations of the vascular network structural organisation and better understand the functional repercussions for the muscle tissue, the disease expression and therapy perspectives using confrontation between morphological (histology and morphometry) and functional (multiparametric and functional nuclear magnetic resonance (mpf-NMR)) approaches.

Taking advantage of $Flk1^{GFP/+}$ mice (all endothelial cells of blood vessels express GFP) crossed with *mdx* mice, we demonstrated for the first time in 3D that the vascular system displayed marked alterations in 12 month-old *mdx* mice, more specifically at the capillary scale: higher capillary volume with similar diameter and increased tortuosity. In parallel, we analysed the repercussions of these disruptions on skeletal muscle physiology, using a non-invasive mpf-NMR protocol consisting in simultaneous acquisition of arterial spin labelling imaging of perfusion and ^{31}P spectroscopy of phosphocreatine kinetic. After a hypoxic stress, even if *mdx* mouse muscles displayed an increase in capillary density, our results demonstrated that blood

perfusion was decreased, although this had no repercussion on energetic parameters. Thus, against expectations, no parallel could be established between capillary density and perfusion. Rather, alterations of capillary organisation *per se,* appeared to impact the phenotypic expression of the disease.

VIII. Smooth muscle

Chairs: Geerten van Nieuw Amerongen and Sarah George.

VIII.O1.

Wnt signaling in vascular smooth muscle cells

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Altered behavior of vascular smooth muscle cells (VSMCs) plays an important role in cardiovascular diseases including atherosclerosis and restenosis. Accelerated VSMC proliferation and migration lead to thickening of the intima and enhanced VSMC apoptosis leads to atherosclerotic plaque rupture and clinical symptoms of atherosclerosis. Consequently, the underlying mechanisms for this altered VSMC behaviour is of great interest. It is well established that the Wnt signaling pathways are critical for embryogenesis and development. In recent years an important role of the Wnt signalling pathways in vascular disease has emerged and is supported by observations of essential Wnt signalling in vascular development. In our laboratory we have investigated the role of Wnt proteins in the regulation of VSMC behaviour. We have demonstrated that Wnt proteins enhance intimal thickening. Interestingly, Wnts have differing effects on VSMCs; Wnt4 promotes proliferation, whilst Wnt2 augments migration. Moreover, we have identified that Wnt proteins act as survival factors for VSMCs and retard VSMC apoptosis at least in part by the up-regulation of WISP-1 (Wnt-inducible secreted protein-1). In summary, it is apparent that Wnts have divergent effects on VSMC behaviour and a greater understanding of Wnt pathways may reveal new therapeutic targets for vascular disease.

VIII.O2.

Contraction of the airway smooth muscle cell in asthma

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Asthma, a common clinical syndrome affecting around 1 in 15 people worldwide, is typified by excessive narrowing of the airways. The end-effecter of excessive airway narrowing is airway smooth muscle (ASM) contraction. Overcoming ASM contraction is therefore a prominent therapeutic strategy, with beta-2 agonists being the main drugs directed at smooth muscle relaxation. Despite their widespread usage, excessive airway narrowing remains poorly controlled in a significant minority of individuals, especially severe asthmatic patients. Promising new ASM relaxant drug candidates have been suggested but a major impediment in their development is the absence of biological screens that can evaluate ASM contraction in a serialized, high-throughput fashion. In the absence of direct measurements of ASM contraction, current biological screens have relied upon correlative changes in calcium signaling, cytoskeletal structure, or focal adhesion composition. These changes can be misleading, however, and are known to frequently yield results opposite to those predicted. Moreover, because of the ubiquitous and multifaceted nature of calcium signaling, specific cell mechanical effects are difficult to deduce from correlative measurements. To overcome these limitations, we introduce Cytoply, a high-throughput technique which incorporates dozens to hundreds of independent measurements of ASM contractile force per experiment. Using Cytoply, we provide measurements of both contractile and relaxation responses of ASM to known compounds. Beyond asthma, Cytoply is applicable to any disease in which contraction of the cell plays a significant role, including acute lung injury, bladder dysfunction, fibrosis, and cancer.

VIII.O3.

Nitric oxide synthase inhibition, with and without inhibition of prostaglandins, reduces blood flow similarly in different human skeletal muscles

I. Heinonen[1,2,5], B. Saltin[3], J. Knuuti[1], Y. Hellsten[4], K. Kalliokoski[1]

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Animal studies suggest that the inhibition of nitric oxide synthesis (NOS) affects blood flow differently in different skeletal muscles, according to their muscle fibre type composition (oxidative vs glycolytic). Quadriceps femoris muscle consists of four different muscle parts: m. vastus intermedius (VI), m. rectus femoris (RF), m. vastus medialis (VM), and m. vastus lateralis (VL). VI muscle is located deep within the muscle group and is generally regarded to consist mostly of oxidative muscle fibres. Consequently, we studied the effect of NOS inhibition on blood flow in these four different muscles by positron emission tomography in eight young healthy men at rest and during one leg dynamic exercise, with and without combined blockade with prostaglandins. At rest blood flow in the VI (2.6 ± 1.1 ml/100 g/min) was significantly higher than in VL (1.9 ± 0.6 ml/100 g/min, $p = 0.015$) and RF (1.7 ± 0.6 ml/100 g/min, $p = 0.0015$), but comparable to VM (2.4 ± 1.1 ml/100 g/min). NOS inhibition alone or with prostaglandins reduced blood flow by almost 50 % ($p < 0.001$), but decrements were found to be similar in all four muscles (drug $\times$ muscle interaction in two-way ANOVA, $p = 0.43$). During exercise blood flow was also the highest in VI (45.4 ± 5.5 ml/100 g/min) as compared to VL (35.0 ± 5.5 ml/100 g/min), RF (38.4 ± 7.4 ml/100 g/min), and VM (36.2 ± 6.8 ml/100 g/min). NOS inhibition alone did not reduce exercising blood flow ($p = 0.51$), but it was reduced significantly ($p = 0.002$), and similarly in all muscles (drug $\times$ muscle interaction, $p = 0.99$) in response to combined NOS and prostaglandin inhibition. In conclusion, NOS inhibition, with or without prostaglandins inhibition, affects blood flow similarly in different human skeletal muscles.

VIII.O4.

Vascular reactivity in pulmonary arterial hypertension (PAH): role of myosin light chain phosphatase and nonmuscle myosin

E. Konik, Y.S. Han, F.V. Brozovich

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PAH is associated with refractory vasoconstriction and impaired NO-mediated vasodilatation. Vascular tone is regulated by light chain (LC) phosphorylation of both nonmuscle (NM) and smooth muscle

(SM) myosin, which is determined by the activities of MLC kinase and MLC phosphatase (MLCP). MLCP is regulated by its myosin targeting (MYPT1) subunit. Alternative splicing produces leucine zipper positive and negative (LZ+/) MYPT1 isoforms, and a LZ+ MYPT1 isoform is required for NO-mediated MLCP activation. We determined the pulmonary arterial contractile phenotype and vascular reactivity in the monocrotaline rat model of PAH. In severe PAH, compared to controls, there was a decrease in relative LZ+ MYPT1 (100 ± 16 vs. 45 ± 6 %, $n = 4$–6), and an increase in NM myosin expression (15 ± 4 vs. 53 ± 5 % of total myosin, $n = 4$–6). The contractile response was also altered in PAH. For KCl depolarization, there was a slowing of both force activation and relaxation. For AngII, the contractile response in controls demonstrated a rapid increase in force to a peak with a subsequent fall to a lower steady state (phasic response), while in PAH force slowly rose to a steady state (tonic response). Further in PAH, the sensitivity to ACh mediated relaxation was reduced. These results demonstrate that the pulmonary arterial contractile phenotype is altered in PAH; the decrease in LZ + MYPT1 expression would contribute to decrease in the sensitivity to NO-mediated vasodilatation, while the increase in NM myosin expression would produce a resting vasoconstriction, and thus the changes in the pulmonary contractile phenotype contribute to the molecular mechanism for PAH.

Tuesday 24 September 2013

Point—Counterpoint
Ca^{2+}-perturbations versus sarcomere dysfunction in cardiac disease.
What matters most? *Moderator*: Karin Sipido.

PC.O1

Point: Ca^{2+}-perturbations cause cardiac dysfunction

D. Eisner

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I will try to persuade you that disordered calcium regulation is much more important than sarcomeric proteins in cardiac disease. In order to do this, I will make use of a variety of arguments; some reasonable and others entirely bogus.

PC.O2.

Counterpoint: sarcomeric dysfunctions are the cause of cardiac dysfunction

R. J. Solaro

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Studies carried out for many years have established that persistent and maladaptive alterations in cellular Ca^{2+}-fluxes are a major mechanism of altered cardiac mechanical, electrical, and metabolic homeostasis. However, a relatively newer body of work has determined that rather than altered Ca^{2+}-fluxes to and from the sarcomeres as a mechanism, a number of cardiac disorders occur with no change in cellular Ca^{2+}-fluxes and are more likely attributable to altered response of the sarcomeres to Ca^{2+}. It is not surprising, that many studies report cardiac disorders with maladaptive defects in both Ca^{2+}-fluxes and sarcomeric response to Ca^{2+}. An unsolved question is the following: What is the most significant maladaptive effect, which happens first in response to common cardiac stresses and triggers the series of events ending in sudden death or progressive heart failure. Our hypothesis has been that the triggering involves altered sarcomeric response to Ca^{2+}, and/or altered sarcomere dynamics, and/or altered signaling at the level of mechano-transduction at Z-disks and M-lines. Strong evidence for this hypothesis is the identification of mutations in proteins of the sarcomere that are linked to common dilated and hypertrophic cardiomyopathies and sudden death. In this case there is no doubt a defect in the sarcomere is causal. Moreover, these mutations induce cellular remodeling of Ca^{2+}-flux regulation and metabolism that are often considered as the primary cause of cardiac disorders.

IX. Mutations in inherited cardiomyopathies

Chairs: Tjeerd Germans and Michelle Michels.

IX.01.

Sarcomere mutations in hypertrophic cardiomyopathy

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Hypertrophic cardiomyopathy (HCM) is a primary heart muscle disease characterized by unexplained left ventricular hypertrophy (LVH)—increased LV wall thickness which occurs in the absence of other causes, such as pressure overload. Sarcomere mutations are an important cause of HCM and are found in up to 70 % of patients. From a clinical perspective, HCM is a complex and heterogeneous disease with remarkable diversity in age of onset, cardiac morphology, and severity of symptoms. In an important subset of patients, HCM is associated with dramatic and important clinical sequelae, including end stage heart failure and increased risk of sudden death. However, the molecular pathways that lead from mutation to clinically overt disease are not well understood. One strategy to gain insight into pathogenesis is studying sarcomere mutation carriers before they develop diagnostic clinical features, specifically cardiac hypertrophy. With this approach, the early manifestations of sarcomere mutations can be identified, free of the confounding influences caused by the dramatic morphologic and functional alterations present in established disease. Such investigations have identified several early phenotypes, including impaired left ventricular relaxation, altered cardiac energetics, and a profibrotic myocardial substrate. Intrinsic differences in contractile performance have also been demonstrated in sarcomere mutation carriers at risk for HCM compared to those at risk for developing dilated cardiomyopathy. With further knowledge, we will begin to better understand how these mutations remodel the heart and begin to develop novel therapies aimed at correcting and preventing disease development in HCM.

IX.02.

Role of miRNA in inherited cardiomyopathies

Y. Pinto

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IX.03.

The ß-myosin mutation R453C in Familial Hypertrophic Cardiomyopathy leads to altered crossbridge kinetics

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Mutations in the heavy chain of ß-myosin (ß-MyHC), the ventricular myosin isoform in humans, are associated with Familial Hypertrophic Cardiomyopathy (FHC). FHC is the most common inherited disease of the heart. Myosin transforms chemical energy from ATP hydrolysis into mechanical work, namely muscle shortening or isometric force generation. Therefore, it is assumed that myosin mutations impair the cross-bridge cycle of the cardiac muscle leading to FHC. However, the underlying molecular mechanisms how individual mutations trigger development of the FHC phenotype are still largely unknown.

To gain insight into possible mechanisms and to test the role of specific subdomains of the myosin head for acto-myosin function, we have analyzed the effects of ß-myosin-mutation R453C on contractile properties of muscle fibers of an FHC patient. This mutation has been reported to cause a severe disease phenotype with an early onset. In accordance, the patient in our study was diagnosed with NYHAII at the age of 19. A biopsy from the *Musculus soleus,* that also expresses the ß-myosin in the slow (Type I) fibers, was taken and used for subsequent analysis.

Interestingly, relative quantification of mutated and wildtype ß-MyHC mRNA from the heterozygous patient revealed a rather low fraction of the R453C mRNA of approximately 30 %. We have shown previously that malignant disease courses of FHC are often correlated with high ratios of mutated mRNA and protein. Since in the case of R453C low amounts of mutated protein induce a malignant phenotype, we assume that this mutation has a severe effect on ß-myosin function. Single fiber biomechanical analysis showed that isometric force generation was increased in the R453C-patient as compared to healthy controls. Fiber stiffness in rigor was not affected by the mutation. This indicates that R453C does not cause a change in stiffness of the myosin heads which could account for the increased force. On the other hand, force generation can be affected by alterations of cross-bridge cycling kinetics. We found that the R453C-mutation leads to significantly higher ATPase activity and an increased rate constant of force redevelopment (k_{tr}) of the fibers. Also, preliminary analysis of in vitro motility showed a tendency to higher gliding velocity of actin on myosin isolated from the patient's muscle fibers.

In summary, our findings indicate that the R453C mutation in the ß-MyHC alters cross-bridge cycling kinetics. It causes higher isometric force generation most likely through an increase in f_{app}, the rate constant of the transition of the cross-bridges into the force generating states. Therefore, residue R453, which is located at the outer end of the nucleotide binding pocket of the ß-myosin head domain, seems to be involved human ventricular myosin ATPase activity. The disturbance of this activity by the R453C-mutation may be the initial trigger to impair the physiological function of the cardiac muscle sarcomere, finally leading to FHC.

IX.04.

Calcium handling in the ACTC E99 K transgenic mouse model of hypertrophic cardiomyopathy (HCM)

C. Rowlands, S.B. Marston, W. Song, K.T. MacLeod

Imperial College, National Heart and Lung Institute, Myocardial Function, London, United Kingdom

Sudden cardiac death (SCD) due to arrhythmias is a significant risk in HCM, but how this arises from a myofilament mutation remains unknown. The E99K transgenic (TG) mouse reproduces many aspects of HCM seen in humans, including a high probability of SCD at 28–45 days, particularly in females. Previous studies demonstrated that the principal effect of this mutation is increased myofilament Ca^{2+}-sensitivity. We hypothesise that this increased sensitivity will disturb intracellular Ca^{2+}-homeostasis, alter the function of key regulatory proteins and cause aberrant electrophysiology.

We isolated ventricular myocytes from E99K TG and non-transgenic (NTG) mice at 2 time points; 25–45 days (young) and 8–12 weeks (old). Cells from young TG mice showed greater sarcomere shortening and faster relaxation than their NTG littermates. In contrast, cells from old TG mice showed prolonged relaxation with no differences in sarcomere shortening. Cardiomyocytes were then loaded with Fura-2AM for measurement of Ca^{2+}-transients. Cells from young TG mice showed increased Ca^{2+}-transient amplitude compared with NTG, but no differences in SR load. Conversely, cells from old TG mice showed smaller Ca^{2+}-transient amplitude and lower SR load than NTG. In both age groups, cells from TG mice displayed slower efflux of Ca^{2+} via the Na^{+}/Ca^{2+}-exchanger but there were no differences in SR Ca^{2+}-ATPase function.

These data show the cardiomyocytes from young TG mice are hypercontractile which, coupled with increased Ca^{2+}-transient amplitude, may increase the propensity for spontaneous Ca^{2+}-release and the formation of afterdepolarisations, predisposing the heart to potentially fatal arrhythmias.

IX.05.

***TTN* mutations are common in families with peripartum cardiomyopathy and dilated cardiomyopathy**

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Background Peripartum cardiomyopathy (PPCM) can be an initial manifestation of familial dilated cardiomyopathy (DCM). We aimed to identify mutations in families that could underlie their PPCM and DCM.

Methods and results We collected families with PPCM and DCM cases from various parts of the world. We studied the clinical characteristics of the PPCM patients and affected relatives, and applied a targeted next-generation sequencing (NGS) approach to detect mutations in 48 genes known to be involved in inherited cardiomyopathies. We identified potentially causal mutations in 11/18 families (61 %) (4 pathogenic mutations and 7 variants of unknown clinical significance which are likely to be pathogenic): 8 in *TTN*, 1 in *BAG3*, 1 in *TNNC1*, and 1 in *MYH7*. Only 1/23 PPCM cases in these families showed recovery of left ventricular function.

Conclusions Targeted NGS shows that potentially causal mutations in cardiomyopathy-related genes are common in families with both PPCM and DCM. This supports the earlier finding that PPCM can be part of familial DCM. Our cohort is characterized by a high proportion of *TTN* mutations and a low recovery rate in PPCM cases.

IX.06.

E163R cardiomyopathy-related cTnT mutation affects Ca^{2+}-regulation and energetics of cardiac myofilaments in a murine model

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Introduction Mutations in cardiac troponin T (cTnT) are the most frequent thin filament protein mutations linked to cardiomyopathies. Here, the impact of the E163R mutation in cTnT on contractile function and tension cost was investigated using skinned ventricular trabeculae from WT and transgenic mouse hearts.
Methods Right and left ventricular trabeculae (194 ± 2.9 μm in width, 173 ± 2 μm in depth, 1.5 ± 0.1 mm in length, $n = 14$) were dissected from WT and E163R mouse hearts and skinned overnight in relaxing solution plus 0.05 % Triton X-100 at 4 °C. Myofibrillar ATPase activity and the relationship between ATP consumption and isometric tension (tension cost) were measured by fluorimetric enzyme coupled assay.
Results No significant differences were found in maximum Ca^{2+}-activated tension of E163R and WT skinned trabeculae (49.96 ± 10.75 mN/mm^2, $n = 6$ and 48.17 ± 7.79 mN/mm^2, $n = 8$ in E163R and WT, respectively). However, Ca^{2+}-sensitivity of tension was significantly increased in E163R skinned trabeculae when compared with WT ($pCa50 = 6.05 \pm 0.04$ and 5.87 ± 0.03 in E163R and WT, respectively, $p < 0.05$). As to the economy of force maintenance, preliminary experiments suggest a 50 % increase of tension cost in trabeculae from E163R hearts. Resting ATPase activity also tended to be higher in E163R preparations (about 5 % of increase).
Conclusions The presence of E163R cTnT mutation in cardiac sarcomeres significantly impact myofilament Ca^2-regulation and energetics without affecting maximal force generation.

X. Regulation of cardiac muscle contraction in health and disease

Chairs: Corrado Poggesi and Diederik Kuster.

X.O1.

Role of cardiac myosin binding protein C in the heart and effects of haploinsufficiency/phosphorylation

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X.O2.

Mutations in thin filament proteins—current state

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Mutations in the genes encoding the thin filament components of the cardiac sarcomere cause a wide range of complex cardiomyopathies. Clinical outcomes are largely driven by both the degree of sarcomeric dysfunction at the biophysical level and the timing and magnitude of the cellular response. More recently, results of genetic testing has suggested that sarcomeric protein variants may act as modifiers and represent novel targets for modulating disease progression. In the current study we have used an integrative in silico—in vitro—in vivo approach to directly test the hypothesis that independent isoforms of cardiac Troponin C (cTnT) can significantly alter the pathogenic remodeling of a Asp230Asn (D230 N) Tropomyosin mutation in vivo via an allosterically mediated change in protein dynamics at the overlap domain. A transgenic mouse model expressing 50 % of it's total tropomyosin as the D230 N form exhibited a progressive LV dilatation that was observed by 2 months of age in the absence of significant fibrosis or inflammation. Double mutants expressing the fetal form of cTnT exhibited an accelerated phenotype, consistent with observations in infants carrying the D230N. The D230N tropomyosin mutation is at an f position at the end of the last canonical period and while CD studies did not reveal a significant unwinding of the C-terminus, Molecular Dynamics simulations suggest that the stabilizing effects of cTnT may be altered in the mutant complex. We predict that these interactions may be further disrupted in the presence of the fetal cTnT and cause a more severe ventricular remodeling pattern.

X.O3.

Urban carbon monoxide pollution aggravates heart failure through oxidative stress

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People with heart failure (HF) are particularly sensitive to outdoor air quality as assessed by the increased risk of hospital cardiac readmission and mortality during peak pollution. Here, we explored the mechanisms underlying the increased cardiac sensitivity of HF patients following chronic exposure to carbon monoxide (CO), an ubiquitous environmental pollutant (second-hand smoke, vehicular exhaust, industrial emissions…).
7 weeks after coronary artery ligation (myocardial infarcted MI), rats were exposed to CO in an airtight exposure container for 4 weeks to reproduce air quality variations environmentally relevant.
We found that exposure to CO exacerbated the MI-associated cardiac remodelling, resulting in increased arrhythmias and in vivo contractile dysfunction. The CO damages involved a cellular remodeling of both Ca^{2+}-handling (Ca^{2+}-transient) and contractile machinery properties. The β-adrenergic reserve altered in MI animals was not worsened by chronic CO exposure. The pre-existent mitochondrial oxidative stress in MI rats was more pronounced after CO exposure. Acute antioxidant treatment with N-acetylcysteine reversed some of the MI-induced and CO-induced alterations at the cellular level, therefore suggesting potential beneficial effects of antioxidant strategies. Moderate exercise training performed in standard filtered air attenuated cardiac remodelling and prevented the additional lesions induced by chronic CO exposure.
The present study shows that chronic exposure to low CO levels mimicking urban atmospheric pollution potentiates and worsens HF-associated cardiac remodelling, thus providing an explanation for epidemiological reports. Since oxidative stress is involved in the

deleterious effects of CO pollution, antioxidant therapy or mild exercise could provide cellular protection against pollution effects.

X.O4.

Right ventricular cellular contractile function is altered and differently modified by antiplatelet agents in a rat model of post-ischemic heart failure

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Previously we demonstrated improvement of left ventricular (LV) function by thrombocyte aggregation inhibitor cilostazol (CIL) and sarpogrelate (SAR) in myocardial infarction (MI)-induced heart failure in the rat. In this study we aimed to characterize the cellular contractile performance of right ventricular (RV) cardiomyocytes in the same animal model.

3 weeks after MI rats were treated with 5 mg/kg/day CIL or SAR, as well as vehicle (HF) (sham-operated animals as controls) for 5 weeks. Then, active force, Ca^{2+}-sensitivity (pCa_{50}) and passive force were measured in membrane permeabilized isolated RV and LV cardiomyocytes.

RV weight was higher in HF (0.48 ± 0.04 g, mean ± SEM) than in the Sham group (0.31 ± 0.02 g) ($p < 0.05$). This increase was prevented by CIL (0.37 ± 0.04 g) or SAR (0.36 ± 0.04 g). The active force and pCa_{50} of cardiomyocytes from the failing RV were not different from that of the Sham group (18.7 ± 1.1 kN/m^2, 5.88 ± 0.03 vs. 18.3.1 ± 2.1 kN/m^2, 5.87 ± 0.03), or those after CIL and SAR treatment (19.6 ± 1.8 kN/m^2, 5.90 ± 0.03; 19.7 ± 1.5 kN/m^2, 5.87 ± 0.02, respectively). The passive force was less in RV of HF (2.1 ± 0.2 kN/m^2) than in the Sham group (3.2 ± 0.4 kN/m^2), and CIL did not (2.9 ± 0.4 kN/m^2), only SAR had the potential to increase it (5.5 ± 0.8 kN/m^2). Measurements on LV cardiomyocytes confirmed elevated active force and pCa_{50} in HF compared to Sham (34.9 ± 2.7 kN/m^2, 5.97 ± 0.03 vs. 26.9 ± 1.6 kN/m^2, 5.85 ± 0.03). We found higher titin phosphorylation in the failing RV compared to Sham.

Data suggest altered RV cardiomyocyte structure and function compared to LV in post-MI heart failure. Changes in RV were diversely affected by CIL and SAR. Decreased passive force in the failing enlarged RV is potentially due to increased titin phosphorylation.

X.O5.

Reverse remodelling in Wistar rats after surgical removal of chronic pressure-overload

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Aims Incomplete myocardial reverse remodeling (MRR) is a major determinant for patient worse outcome. We aimed to establish and characterize an animal model that mimics the structural and functional changes in MMR after chronic pressure-overload (PO) relief.

Methods PO was established in male Wistar rats by ascending aortic constriction. 5 weeks later, an echocardiographic evaluation was performed to assess cardiac function. Subsequently, a surgical debanding procedure (Deb) was performed in half of the banding (Ba) and the corresponding control group (Sham), resulting in 2 additional groups: Ba_Deb and Sham_Deb. The cardiac function was monitored weekly by echocardiography and, at the end of the protocol pressure–volume loops were analyzed, active and passive tensions of myofilaments were measured and AGES deposition quantified.

Results Compared to Sham, PO increased LV end-systolic (ESP, Sham = 112.4 ± 6.7, Ba = 168.2 ± 6.7 mmHg, $p = 0.0011$) and end-diastolic pressure (EDP, Sham = 5.3 ± 1.1, Ba = 8.7 ± 0.2 mmHg, $p < 0.0001$), dP/dtmax (Sham = 5627 ± 931, Ba = 8535 ± 390 mmHg/s, $p = 0.0274$) and LVmass (Sham = 0.78 ± 0.01, Ba = 1.10 ± 0.05 g, $p < 0.0001$). Moreover, Ba presented impaired relaxation (τ, Sham = 7.8 ± 0.4, Ba = 9.4 ± 1.1 ms, $p = 0.0468$), increased AGES deposition (Sham = 5.2 ± 0.5, Ba = 10.9 ± 2.0, $p < 0.0001$) and increase passive force (Sham = 5.0 ± 0.5 mN/mm^2, Ba = 6.6 ± 0.7 mN/mm^2 SL = 2.3 μm, $p < 0.05$). Despite the presence of diastolic dysfunction (DD), ejection fraction was preserved and myofilaments active force was similar between groups. After Deb procedure, we observed similar values among Ba_Deb and Sham_Deb group (ESP: Ba_Deb = 101.3 ± 3.4, Sham_Deb = 102.2 ± 8.2 mmHg; EDP: Ba_Deb = 4.4 ± 0.8, Sham_Deb = 4.9 ± 0.7 mmHg; dP/dtmax: Ba_Deb = 702 8 ± 565, Sham_Deb = 6977 ± 646 mmHg/s; τ: Ba_Deb = 7.1 ± 0.5, Sham_Deb = 7.7 ± 0.4 ms; AGES: Ba_Deb = 5.75 ± 0.20, Sham_Deb = 6.43 ± 0.32od). Additionally to Lvmass regression (Ba_Deb = 0.72 ± 0.10, Sham_Deb = 0.71 ± 0.13 g) also passive force trend to equalize sham group.

Conclusions A complete regression of LVmass and DD was observed with PO-removal. We successfully characterized an animal model that mimics aortic stenosis and subsequent aortic valve replacement, opening new avenues for mechanistic and pharmacological studies.

X.O6.

Phosphorylation of protein kinase C sites SER42/44 blunts enhanced length-dependent activation in response to protein kinase A

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Background Evidence suggests that protein kinase C (PKC)-mediated phosphorylation of troponin I (cTnI) at Ser42/44 is increased in heart failure. While studies in rodents demonstrated that PKC-mediated phosphorylation at Ser42/44 decreases maximal force and ATPase activity, PKC incubation of human cardiomyocytes did not affect maximal force. Functional effects of PKC-mediated phosphorylation of Ser42/44 may be species-dependent. Therefore, we investigated whether Ser42/44 pseudo-phosphorylation affects force development and ATPase activity using troponin exchange experiments in human myocardium. Additionally, we studied whether pseudo-phosphorylated Ser42/44 modulates length-dependent activation of force, the cellular basis of the Frank–Starling relation, which is known to be regulated by protein kinase A (PKA)-mediated cTnI phosphorylation at Ser23/24.

Methods Isometric force was measured at various [Ca^{2+}] in membrane-permeabilised cardiomyocytes exchanged with human recombinant wild-type (Wt) troponin or troponin mutated at the

PKC sites Ser42/44, or the PKA sites Ser23/24 into aspartic acid (D) or alanine (A) to mimic phosphorylation and dephosphorylation, respectively. In troponin-exchanged donor cardiomyocytes experiments were repeated after PKA incubation. ATPase activity was measured in troponin-exchanged human muscle strips.
Results Compared to Wt, 42D/44D decreased Ca^{2+}-sensitivity without affecting maximal force in failing and donor cardiomyocytes. In donor myocardium, pseudo-phosphorylation of Ser42/44 did not affect maximal ATPase activity or tension cost, however, blunted length-dependent increase in Ca^{2+}-sensitivity induced upon PKA-mediated phosphorylation of cTnI-Ser23/24.

Wednesday 25 September 2013

XI. Muscle physiology: extreme functional demands

Chairs: Willem van der Laarse and Coert Zuurbier.

XI.O1.

Systems biology of muscle mitochondria

R. Balaban

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XI.O2.

Cardiac and skeletal muscle metabolism in high altitude hypoxia

A. J. Murray

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In most tissues of the body, cellular ATP production predominantly occurs via mitochondrial oxidative phosphorylation of reduced intermediates, which are in turn derived from substrates such as glucose and fatty acids. In order to maintain ATP homeostasis, and therefore cellular function, the mitochondria require a constant supply of fuels and oxygen. In many disease states, or in healthy individuals at altitude, tissue oxygen levels fall and the cell must meet this hypoxic challenge to maintain energetics and limit oxidative stress. For oxidative tissues such as heart or skeletal muscle, cellular hypoxia necessitates changes in gene and protein expression that alter mitochondrial function. It is known that lowlanders returning from high altitude have decreased muscle mitochondrial densities, yet the underlying transcriptional mechanisms and time course were not fully understood. To explore these, we measured gene and protein expression plus ultrastructure in muscle biopsies of lowlanders at sea level and following exposure to hypobaric hypoxia. Subacute exposure (19 days after initiating ascent to Everest base camp, 5300 m) was not associated with mitochondrial loss. After 66 days at altitude and ascent beyond 6,400 m, mitochondrial densities fell by 21 %, with loss of 73 % of subsarcolemmal mitochondria. Correspondingly, levels of the transcriptional coactivator PGC-1α fell by 35 %, suggesting down-regulation of mitochondrial biogenesis. Sustained hypoxia also decreased expression of electron transport chain complexes I and IV and UCP3 levels. We suggest that during subacute hypoxia, mitochondria might be protected from oxidative stress. However, following sustained exposure, mitochondrial biogenesis is deactivated and uncoupling down-regulated, perhaps to improve the efficiency of ATP production.

XI.O3.

Epinephrine restrains the fasting-induced catabolic effects on skeletal muscle protein breakdown

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The physiological role of epinephrine in the regulation of skeletal muscle protein metabolism under fasting conditions is unknown. We examined the effects of plasma epinephrine depletion, induced by adrenodemedullation (ADMX), on muscle protein metabolism in fed and 2-day fasted rats. In fed rats, ADMX for 10 days reduced muscle mass, the cross-sectional area of extensor digitorum longus (EDL) muscle fibers, and the phosphorylation levels of Akt. In addition, ADMX treatment led to a compensatory increase in muscle sympathetic activity, as estimated by the rate of norepinephrine turnover; this increase was accompanied by high rates of muscle protein synthesis. In fasted rats, ADMX treatment exacerbated fasting-induced proteolysis in EDL but did not affect the low rates of protein synthesis. Accordingly, ADMX activated lysosomal proteolysis and further increased the activity of the Ub-proteasome system (UPS). Moreover, expression of the atrophy-related Ub ligases atrogin-1 and MuRF1 and the autophagy-related genes LC3b and GABARAPl1 were up-regulated in EDL muscles from fasted rats, and ADMX reduced cAMP levels and increased fasting-induced Akt dephosphorylation. In isolated EDL, epinephrine reduced the basal UPS activity and suppressed overall proteolysis and atrogin-1 and MuRF1 induction following fasting. These data suggest that epinephrine released from the adrenal medulla inhibits fasting-induced protein breakdown in fast-twitch skeletal muscles, and these anti-proteolytic effects on the UPS and lysosomal system are apparently mediated through a cAMP/Akt-dependent pathway, which leads to suppression of ubiquitination and autophagy.

XI.O4.

Acute regulation of the stars signalling pathway in response to endurance and resistance exercise

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Introduction The striated muscle activator of Rho signalling (STARS) protein may act as a sensor to transduce extracellular mechanical stress signals and activate molecular pathways involved in skeletal muscle growth and metabolism. In two independent studies, we investigated acute responses of STARS signalling pathway components in response to different modes of contraction.
Methods In one study, 18 subjects performed a single bout of resistance or endurance exercise in a training-habituated state (n = 9 per group), with muscle biopsies harvested at 0, 2.5 and 5 h after exercise. In another study, 24 untrained subjects performed a single bout of eccentric or concentric resistance exercise using alternate legs, with muscle biopsies harvested at 1, 3 and 5 h after

exercise. STARS, MRTF-A, and SRF gene and protein expression were measured using real-time PCR and western blotting, respectively.

Results STARS gene expression increased following each type of resistance exercise, with eccentric contraction being a stronger driver than mixed or concentric contraction. MRTF-A gene expression decreased following all modes of exercise. SRF gene expression and SRF protein phosphorylation increased following concentric and eccentric contraction in the untrained state only.

Conclusion STARS provides a link between mechanical stress and downstream signalling and may exert a role in triggering intracellular signals responsible for muscle adaptation to resistance exercise. The greater ability of eccentric resistance exercise to promote the up regulation of STARS and SRF mRNA, when compared to concentric exercise, suggests that the STARS signalling may be involved in muscle regeneration to muscle-damaging exercise.

XI.O5.

Skeletal muscle oxidative function after a 10-day exposure to hypoxia and microgravity

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Skeletal muscle oxidative function was evaluated before (CTRL) and after 10 days of normobaric hypoxic (inspired PO_2 12.5 kPa) bed rest (H-BR) and normoxic bed rest (BR) in nine healthy young men (age: 24.1 ± 1.7 years [mean ± SD]). H-BR was taken as a simulation of lunar habitats.

Pulmonary gas exchange and *vastus lateralis* muscle fractional O_2-extraction (by near-infrared spectroscopy) were determined during an incremental cycle ergometer (CE) and one-leg knee extension (KE) exercise up to volitional exhaustion. Mitochondrial respiration was evaluated by high-resolution respirometry in permeabilized vastus lateralis fibers.

During CE, peak pulmonary O_2-uptake ($\dot{V}O_2$ peak) significantly decreased by ~7 and 9 % after BR and H-BR, respectively. During KE, $\dot{V}O_2$ peak decreased significantly (by ~6 %) after BR (0.83 ± 0.07 vs. 0.89 ± 0.08 [CTRL] L min^{-1}), but not after H-BR (0.86 ± 0.07 vs. 0.90 ± 0.07). Skeletal muscle peak fractional O_2-extraction was significantly lower after BR (60 ± 12 vs. 70 ± 11 % [CTRL]), but not after H-BR (65 ± 12 vs. 71 ± 10). No significant changes were observed in maximal ADP-stimulated mitochondrial respiration either after BR (48.9 ± 11.5 pmol $O_2 \cdot s^{-1}$ mg^{-1} wet weight) or H-BR (42.8 ± 7.8) vs. CTRL (46.6 ± 6.6).

After relieving, by the KE exercise protocol, constraints related to cardiovascular O_2-delivery, oxidative function in vivo was still impaired after BR, while a less pronounced impairment was evident after H-BR. Mitochondrial respiratory function ex vivo was unchanged after both conditions. Peripheral O_2-diffusion constraints and/or an impaired O_2-delivery/O_2-utilization coupling might be the main bottleneck to oxidative function in vivo after BR. Hypoxia may attenuate the impairment of muscle oxidative function observed after BR.

XII. Mitochondrial function in health and disease

Chairs: Ger Stienen and Christoph Maack.

XII.O1.

Contribution of mitochondrial ROS formation to myofibrillar protein oxidation in cardiac and muscle disease

M. Canton

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The intracellular accumulation of reactive oxygen species (ROS) is widely accepted to play a key role in the contractile dysfunction of cardiac and skeletal muscles. However, scarce information is available concerning the molecular mechanisms underlying the contractile impairment and the most relevant sources of ROS.

We recently demonstrated that myofibrillar proteins represent important intracellular targets of ROS in several models of myocardial dysfunction, such as coronary microembolization and heart failure, as well as in two murine models of muscular dystrophy. More in detail, we showed that tropomyosin and actin oxidation correlated with contractile dysfunction, not only in experimental models but also in biopsies from heart failure patients.

The intracellular increase in ROS formation detected under pathological conditions is mainly due to mitochondria. We demonstrated that, besides the respiratory chain, monoamine oxidases (MAO) are a relevant source of ROS. These flavoproteins, located in the outer mitochondrial membrane, catalyze electron transfer from biogenic amines to oxygen, producing large amounts of H_2O_2. MAO-dependent ROS accumulation plays a crucial role in determining both loss of viability, due to mitochondrial dysfunction, and contractile impairment, due to oxidative alterations of the myofibrillar proteins. In particular, MAO inhibition prevented myofibrillar protein oxidation ameliorating biochemical, histological and functional derangements in models of muscle dystrophy. These findings complement the protective actions elicited by pharmacological and genetic inhibition of MAO that we documented in several models of cardiac injury.

In conclusion, we showed that the intracellular accumulation of ROS following mitochondrial dysfunction induces oxidative modifications of the myofibrillar proteins contributing to the contractile impairment of the cardiac and skeletal muscle.

XII.O2.

Regulation of mitochondrial ROS formation in cardiac myocytes

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Reactive oxygen species (ROS) play a critical role in the development and progress of chronic heart failure. A major source of ROS are mitochondria, where superoxide is generated at the electron transport chain. After dismutation of superoxide to H_2O_2, the latter is eliminated by antioxidative enzymes that require NADPH. NADPH is regenerated by enzymes that derive their substrates from the Krebs cycle. Since the Krebs cycle is controlled by Ca^{2+}, mitochondrial Ca^{2+} uptake is important not only to match energy supply to demand, but also to prevent ROS emission from mitochondria. Besides regulation through Ca^{2+}, the redox state of NADH and NADPH is

controlled importantly by mechanical work performed by the myofilaments and ion handling proteins. Hence, load conditions of the heart (and thus, myofilaments) have an important impact on mitochondrial ROS production. In my talk, I will discuss the mechanisms that regulate mitochondrial ROS production in response to physiological and pathological cardiac workload. These mechanisms may represent targets for the treatment of heart failure.

XII.O3.

TNF-α-mediated caspase-8 activation induces ros production and trpm2 activation in adult ventricular myocytes

S. Roberge[1], J. Roussel[1], D.C. Andersson[2], B. Vidal[1], J. T. Lanner[2], A. Katz[2], H. Westerblad[2], A. Lacampagne[1], J. Fauconnier[1]

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TRPM2 is a Ca^{2+}-permeable cationic channel of the transient receptor potential (TRP) superfamily that is linked to apoptotic signaling. Its involvement in cardiac pathophysiology is unknown. The aim of this study was to determine whether the pro-apoptotic cytokine tumor necrosis factor-α (TNF-α) induces a TRPM2-like current in murine ventricular cardiomyocytes. Adult isolated cardiomyocytes from C57/Bl6 mice were exposed to TNF-α (10 ng/mL). Western-blotting showed TRPM2 expression, which was not changed after TNF-α incubation. Using patch-clamp in whole cell configuration, a non-specific cation current was recorded after exposure to TNF-α (ITNF). ITNF was inhibited by the caspase-8 inhibitor z-IETD-fmk, the antioxidant N-acetylcysteine, and the TRPM2 inhibitor clotrimazole. TRPM2 has previously been shown to be activated by ADP-ribose, which is produced by poly (ADP-ribose) polymerase 1 (PARP1). TNF-α exposure resulted in increased poly-ADP-ribosylation of proteins and the PARP-1 inhibitor 3-aminobenzamide inhibited ITNF. TNF-α exposure increased the mitochondrial production of reactive oxygen species (ROS; measured with the fluorescent indicator MitoSOX Red) and this increase was blocked by the caspase-8 inhibitor z-IETD-fmk. TRPM2 inhibition with clotrimazole decreased TNF-α-induced cardiomyocyte death and the infarct size in heart following ischemia–reperfusion. These results demonstrate that TNF-α induces a TRPM2 current in adult ventricular cardiomyocytes. TNF-α induces caspase-8 activation leading to ROS production, PARP-1 activation and ADP-ribose production. TNF-induced TRPM2 activation contributes to cardiomyocytes cell death and reperfusion injury.

XII.O4.

Reduced expression of creatine kinase in rat failing right ventricle causes diastolic dysfunction in ventricular myocytes

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Right ventricle (RV) function is a prognostic indicator of mortality in patients with pulmonary arterial hypertension, yet the underlying causes of contractile dysfunction are not fully understood. We wished to test whether reduced expression of creatine kinase (CK) in rats with RV failure is associated with diastolic dysfunction in ventricular myocytes.

200 g male Wistar rats were injected with 60 mg kg^{-1} monocrotaline to induce RV failure (MCT), or saline as a control (CON). MCT rats were sacrificed upon showing signs of heart failure, the heart removed and ventricles either homogenised for Western blot analysis or dissociated into isolated myocytes.

Western blot analysis revealed muscle-isoform CK was reduced by 50 % in RV of MCT rats vs CON ($n = 6$, $p < 0.05$). There was a shorter diastolic sarcomere length (SL) in MCT RV myocytes (MCT 1.79 ± 0.01 μm, CON 1.89 ± 0.01 μm, $p < 0.05$, $n = 79–139$) that was Ca^{2+}-independent. The CK inhibitor DNFB (20 μM) shortened diastolic SL in CON (1.69 ± 0.02 μm) and MCT myocytes (1.67 ± 0.02 μm) ($p < 0.05$, $n = 24$). CK reversibly binds to the myofilaments in saponin-skinned muscle fibres; skinned MCT SL was shorter than CON (MCT 1.83 ± 0.02 μm, CON 1.95 ± 0.01 μm, $p < 0.05$, $n = 24$). However, incubating skinned MCT myocytes with CK (4.4 mg.ml^{-1}, 30 min) and 10 mM phosphocreatine restored SL to CON length (FAIL 1.90 ± 0.01 μm, $p > 0.05$, $n = 24$).

We conclude reduced CK expression in failing RV causes diastolic dysfunction through altered crossbridge cycling and are investigating the functional consequences in isolated myocytes and whole-hearts.

XII.O5.

NADH and FAD kinetics reveal altered mitochondrial function in right ventricular heart failure

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In chronic heart failure, alterations occur in cardiac metabolism, enzyme content calcium handling and mitochondrial function. Little is understood about the influence of alterations in mitochondrial function on energy metabolism during the transition from cardiac hypertrophy (CH) to failure (HF). A photometry-based technique was used to simultaneously measure contractile function and autofluorescence of NADH (reduced nicotinamide adenine dinucleotide) and FAD (oxidized flavin adenine dinucleotide) in thin cardiac trabeculae. Three groups were studied: CH (monocrotaline: 40 mg kg^{-1}) or HF (60 mg kg^{-1}) and CON (saline). After 23 ± 1 days, right ventricular trabeculae were excised, attached to a force transducer and superfused with oxygenated tyrode with 1 mM Ca^{2+}. Autofluorescence of both NADH (excitation: 340 nm; emission: 470 nm) and FAD (450/525 nm) were recorded using an inverted microscope during transitions in pacing frequency between 0.5 and 3 Hz at 27 °C. Upon an increase in pacing frequency, force–time integral increased less in CH and HF compared to CON. In CON, steady-state NADH concentration at 3 Hz was lower than at 0.5 Hz. Steady-state NADH concentration however was higher than at 0.5 Hz in CH and increased further in HF. FAD responses showed asymmetrical alternations in CH, suggestive of complex I dysfunction in CH. Despite a smaller increase in force–time integral, the initial changes in NADH and FAD were more pronounced in CH and HF than in CON, indicating a larger deviation in metabolic homeostasis in CH and HF. These results are suggestive of a mismatch between dehydrogenase activity and oxidative phosphorylation, and reveal an altered mitochondrial complex function in CH and HF. This work is supported by CVON2011-11 ARENA.

ABSTRACTS—POSTERS PER SESSION AND DAY

Sunday September 22 2013

POSTER SESSION I Mechanotransduction/muscle adaptation

I.P1

The *compact* mutation of myostatin causes a glycolytic change in the phenotype of skeletal muscles

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Myostatin is an important negative regulator of skeletal muscle growth. *Compact (Cmpt)* mice, similar to the myostatin knock out animals, are characterized by hypermuscularity. The *Cmpt* mutation comprises a 12-bp deletion in the propeptide region of myostatin, however, modifier genes are also involved in the full penetration of the phenotype. Previously, we demonstrated that hypermuscularity of *Cmpt* mice was exclusively caused by fiber hyperplasia without any hypertrophy.
Aims To analyze the possible effects of the *Cmpt* mutation on the fiber type composition, immunostaining of the different Myosin Heavy Chain (MHC) isoforms was carried out in the tibialis anterior (TA) muscles of 10–12 week old male *Cmpt* and wild type (BALB/c) mice. Moreover, transcript levels of MHC isoforms was investigated by isolating total RNA from the contralateral TA muscles followed by qRT-PCR analysis using Hprt (hypoxantine guanine phosphoribosyl transferase) as an internal control.
Results Immunohistochemical analysis revealed a significant increase in the number of the glycolytic IIB fibers (80.3 % ± 3.3 vs. 50.3 % ± 3.3, mean ± SEM) while a significantly decreased number of the more oxidative IIX and IIA fibers (IIX: 19 % ± 3.2 vs. 46.4 % ± 2.6, IIA: 0.65 % ± 0.05 vs. 3.3 % ± 0.09) was present in *Cmpt* mice showing a glycolytic shift in the muscle phenotype compared to the wild type. Similarly, MHCIIB mRNA levels were significantly increased, while MHCIIX and IIA transcript levels significantly decreased in *Cmpt* animals compared to BALB/c ones. These results suggest that the glycolytic shift in TA muscle is primarily regulated at the level of MHC transcription.

I.P2.

Increased mass rather than specific tension causes the increased force generating capacity in hypertrophied adult and old mouse plantaris muscle

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Resistance exercise is an effective means to counteract the age-related loss in muscle force and mass. It is often accompanied by both an increase in muscle mass and specific tension. Here we addressed the question to what extent an increase in muscle mass and specific tension contribute to the improvement in muscle function after a hypertrophic stimulus. To address this question we overloaded the left plantaris muscle of male 9- ($n = 11$) and 25-month-old ($n = 10$) C57Bl/6j mice by denervation of the synergists. After 6 weeks overload the maximal isometric force and mass were increased with ~30 % in both groups ($p < 0.01$). Specific tension, muscle length and fibre number did not differ between conditions ($p > 0.05$). The fibre type shift from type IIB to type IIA and IIX with overload ($p < 0.01$) was larger for adult (50–27 % IIB) than old muscle (42–38 % IIB; age × overload interaction; $p < 0.01$). Furthermore, old muscle had a lower proportion of hybrids in control muscle, but a larger increase in hybrids after overload, compared to adults (age × overload interaction; $p < 0.01$). Stepwise regression indicated that most of the increased maximal muscle force was attributable to an increase in muscle mass (67 %) for both groups, and also specific tension (23 %) in the old. In conclusion, the hypertrophic response is not attenuated in 25-month-old mice. However, it seems that shifts in fibre type proportions were blunted in the old. The main gain in force generating capacity in overloaded muscles is thus due to an increase in muscle mass, rather than an increase in specific tension.

I.P3.

Quantification of limb neuromuscular activation via viscoelastic property mapping with magnetic resonance elastography

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Introduction Viscoelastic properties of muscle tissue reflect shifts in tissue microstructure including muscle stretch and contraction. Magnetic resonance elastography (MRE) allows these mechanical properties to be determined in vivo without limitations on depth. Here MRE was used to map the viscoelastic properties of the thigh muscles during conditions of rest and knee extension.
Methods Eleven subjects were scanned in a Siemens Verio 3T magnet during conditions of supine rest and supine knee extension. 50 Hz acoustic waves were simultaneously propagated through the tissue using a subwoofer attached to a ring actuator. The tissue was modelled as transversely isotropic with plane waves propagating in the plane of isotropy. MRE-J software was used to create maps of storage modulus, loss modulus, and power law constant. The maps were manually co-registered and subjected to significance testing with $p < 0.05$.
Results Mean stiffness increase among the significant pixels was 84 % for storage modulus and 105 % for loss modulus. 55 % of the power law significant pixels were an increase in the value and 45 % of the pixels were a decrease in the value.
Discussion The viscoelastic maps captured a group effect of increases in the storage and loss modulus of contracted muscle tissue. The parameter of power law constant appeared to distinguish between muscles that grew stiffer from contraction and muscles that grew stiffer by stretching.
While elastography results are highly dependent on testing methods, statistical mapping allows varied methods of acquisition to report in the common language of significance and effect size.

I.P4.

Non-invasive assessment of muscle function—acoustic myography re-invented

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Assessment of muscle function whether sport or daily home activity related has been confounded due to measuring equipment limitations, problems with noise, and limitations regarding freedom of movement. Acoustic myography (AMG) is a non-invasive, purely mechanical method which until recently has suffered from being not easily applicable, as well as not being able to register muscle movement at a sufficient sampling speed. With modern amplifiers and digital sound recording this has changed.
Our aim was to develop a setup for muscle-sound assessment, which could be reliably applied in any local setting e.g. sports and clinic alike. Two healthy subjects were assessed during standing, stair climbing, walking, and running. Piezo-electric microphones were applied to the skin. A digital sound recorder enabled sampling speeds of 96,000 Hz. Surface electromyography (sEMG) was measured simultaneously during movements and served as a comparison. The recorded signals were assessed and described in terms of their signal frequency (Hz) and peak-to-peak amplitude (mV) using Chart analysis software.
AMG demonstrated a good representation of the work of each muscle group during simple and complex movements, and run in parallel with sEMG, AMG measurements were validated as representing anticipated muscle activity.
The setup was finally tested on a group of runners and on race horses during movement, where recordings gave equally reliable results concerning muscle function, compared to those seen with the subjects tested under laboratory conditions.
In conclusion we feel that our re-invented AMG technique shows great potential for 'real-life' assessment of muscle function and in clinical diagnostics.

I.P5.

Masticatory muscles do not undergo atrophy in space

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Muscle loading is important for maintenance of muscle mass, and when load is removed, atrophy is inevitable. The ultimate anti-gravity environment is in space, where several past experiments documented significant loss in muscle mass. We were given the opportunity to compare the response of masticatory and axial muscles to anti-gravity, using mice aboard the space shuttle mission STS-135 and age and sex matched controls. We hypothesized that the loading of masseter muscles comes in part from normal chewing activity, and so they may be spared from atrophy in antigravity. Mice were subjected to 10 days space flight, and a second cohort remained on the ground. Both sets of mice received the same nutrient upgraded rodent food bars, and there was no significant difference in food intake. At the end of space flight, muscles were dissected from both cohorts of mice, where one masseter and one tibialis anterior were rapidly frozen for biochemical molecular measurements, and the contralateral masseter and 1 extensor digitorum longus were processed for morphological measurements. The limb muscles exhibited a reduction in muscle fiber size, diminished phosphorylation of FAK and Akt, and increased atrogene expression, as observed in previous studies. In contrast, the masseters had no significant change in fiber size, FAK and Akt phosphorylation, or atrogene expression. Thus, continued loading activity in an anti-gravity environment prevents atrophy.

I.P6.

Effects of a 21 day Bed Rest on the PI3/AKT pathway: role of O-GlcNAc

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The effects of the MEP 21-day bedrest (DLR) were investigated on soleus (SOL) and vastus lateralis (VL) biopsies from 10 healthy male subjects before and after bedrest (BR). Control (CTR) subjects were assigned to BR only, NUTR subjects were submitted to BR with a nutrition protocol (supplementation of whey protein and alkaline minerals). Long-term inactivity due to weightlessness conditions results in muscle atrophy, force reduction and phenotype transitions. Thus, we studied the anabolic PI3K-AKT-mTor and the catabolic PI3K-AKT-FOXO signalling pathways, known to play pivotal roles in modulating both muscle protein degradation and synthesis processes. We also analyze the role of O-GlcNAcylation on muscle atrophy and the interplay between O-GlcNAc and phosphorylation in the activation of AKT. Indeed, several factors involved in the PI3K-AKT pathway are modified by *O*-GlcNAcylation.
Our results showed a decrease in the activation of AKT for SOL and VL in both experimental groups. Decrease activation of the anabolic pathway and activation of the catabolic pathway was measured in VL in CTR. The activation of the atrogenes seems reduced in the NUTR group. More heterogeneous effects were observed in SOL.
The global level of O-GlcNAcylation decreased after BR in VL and SOL, as previously described in a rat model of functional atrophy, suggesting a role of this post-translational modification in the development of muscle atrophy in human. A decrease level of AKT O-GlcNAcylation was associated to the decrease of AKT phosphorylation in SOL and VL from CTR. Consequently, O-GlcNAcylation seems not to interfere with AKT phosphorylation but rather acts in a synergic effect.
This work was supported by grants from Centre National d'Etudes Spatiales (SEP 09215).

I.P7.

Treatment with trichostatin A attenuates unloaded-induced skeletal muscle atrophy

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Exposure to reduced activity induces a functional and phenotypic atrophy of skeletal muscle. This study was designed to test the hypothesis that treatment of trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, would partly counteract unloading-induced soleus muscle atrophy. Soleus muscle atrophy (−38 %) induced by 14 days of hindlimb suspension was reduced to only 25 % under TSA treatment. TSA treatment partly prevented the loss of type I (−41 vs. −23 % for H rats) and IIa (−39 vs. −20 % for H rats) fiber size and suppressed the slow-to fast-twitch fiber transitions in soleus muscle. Trichostatin A abolished the decrease in glutathione vs glutathione disulfide ratio and the increase in catalase activity (biomarkers of oxidative stress) observed after unloading. The ATP-ubiquitin proteasome and the autophagy-lysosome pathways are involved in muscle atrophy through an activation of muscle proteolysis. TSA treatment was associated with a slight decrease in the levels of two atrophy-related muscle-specific ubiquitine ligases: muscle ring finger1 (MurF1) and atrogin-1 proteins. No change occurred in LC3b mRNA (autophagosome expansion and completion). Unloading resulted in a significant decrease of Bnip3 (mitophagy) mRNA but TSA treatment failed to modify the downregulation of Bnip3 transcript. The beneficial effect of TSA was not associated with a modulation of the myostatin/follistatin axis. Our findings suggest that

deacetylase inhibitors such as Trichostatin A might be regard as an adjuvant to protect muscle cells from unloaded-induced atrophy.

I.P8.

Compared to 10 mo old mice, 24 mo old male c57/bl6 mice show characteristics of sarcopenia and anabolic resistance

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Ageing is associated with sarcopenia, a progressive decline of skeletal muscle mass, muscle quality and muscle function. Although muscle parameters have been studied in mice of various ages/strains, no single study characterized these parameters in mice of various ages of the same strain. Therefore, male 10 and 24mo old C57BL6/6J mice ($n = 45$/group) were studied for the parameters of sarcopenia and the presence of anabolic resistance as part of its pathophysiology, with the aim of characterizing sarcopenia in old mice. Statistics were performed by ANOVA with post hoc LSD. Muscle mass of the hind limb was lower in 24 mo (426 ± 10 mg) compared to 10 mo (481 ± 8 mg) old mice ($p < 0.05$). A significant decrease in physical daily activity (-62.8 %), muscle grip strength (−11.1 %) and ex vivo muscle contractile function (−22.4 % maximal force production) was observed in 24 mo compared to 10 mo old mice ($p < 0.05$). The muscle anabolic response to a single standard meal was tested and showed increased muscle protein synthesis in young, but not in old mice ($p < 0.05$) indicative of anabolic resistance in the old mice. This study shows clear characteristics of sarcopenia and anabolic resistance in 24 mo old C57/BL6 male mice. These observations offer potential to explore the effects of interventions targeting sarcopenia in the future using this mouse model.

I.P9.

Muscle damage after neuromuscular electrostimulation assessed by multimodal magnetic resonance imaging

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Neuromuscular electrostimulation (NMES) is useful for improving muscle function. However, it has been reported that NMES induces muscle damage mainly due to the specificity of motor unit recruitment (i.e., non-selective, spatially fixed, synchronous and mainly superficial) so that one could expect heterogeneous damage between muscles. NMES-induced muscle damage was quantified by multimodal magnetic resonance imaging (MRI) including T_2 mapping and diffusion tensor imaging (DTI) for the quadriceps muscles.
Eleven young male subjects took part in a single bout of NMES with the stimulation electrodes being placed over the belly of *vastus lateralis* [VL] and *vastus medialis* [VM]. Changes in T_2 values and DTI metrics (diffusivities) were assessed in thigh muscles (i.e. VL, VM, *rectus femoris* [RF] and *vastus intermedius* [VI]) before and 4 days after the NMES session using a 1.5T scanner.
Mean increases in T_2 were larger for the VL (+17.7 %) and VM (+8.0 %) when compared with the VI (+3.5 %) muscle. Diffusivities increased for the VL [2.1–5.8 %], VM [1.7–3.0 %] and VI [1.1–2.0 %] without difference among them. Both T_2 values and diffusivities remained unchanged for the RF.
The extent of muscle damage was greater for the directly stimulated muscles than indirectly stimulated muscles and might be related to the activation of deep intramuscular nerve branches and/or shear stress between muscles. The extent of changes varied between T_2 and DTI metrics suggesting implication of different physiological processes and highlighting the relevance of multimodal MRI for monitoring in vivo skeletal muscle injury.

I.P10.

Tenascin-C is quantitatively related to angiotensin-modulated muscle growth in man

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Mechano-regulated expression of the modular extracellular matrix protein Tenascin-C orchestrates muscle repair by the stimulation of myogenesis, which is understood to be promoted by angiotensin converting enzyme (ACE) inhibition. We have tested the hypothesis that exercise induces the expression of Tenascin-C content and that this would correspond to ACE-modulated changes in cross sectional area of muscle fibers in men.
Tenascin-C was detected as a large (250 kDa) and small (180 kDa) isoform in vastus lateralis muscle. Bicycle type endurance training elevated the content of both, the small and large tenascin-C isoform twofold ($p = 0.05$), in vastus lateralis muscle ($n = 17$). Microarray analysis identified Tenascin-C transcript levels as training-induced target of genetic inhibition of ACE activity by the presence of the ACE I-allele (30 % difference between ACE-DD vs. ACE-ID genotype, statistical analysis of microarrays, q value = 2.8 %). Pharmacological inhibition of angiotensin 2 production by ACE reduced mean cross sectional area of muscle fibers (−30 %, $p = 0.07$) in line with a reduction of the training-induced transcript expression of type I and IIa myosin heavy chain (−45 %, $p < 0.05$) and Tenascin-C (−60 %, $p < 0.05$).
The findings motivate a mechanism whereby tenascin-C expression is a component of the adaptive growth response of muscle fibers to moderate forms of mechanical loading.

I.P11.

Gene expression in masseter muscle after orthognathic surgery in comparison with untreated healthy controls

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Deficiency in functional adaptation after Orthognathic surgery could be one reason for relapse. Aim of the study was to compare gene expression in masseter muscle between untreated controls and patients before and after orthognathic surgery.
50 adult patients (mean age 23.6 years) divided in groups with mandibular prognathia (15), retrognathia (20) and untreated controls (15) were involved. Tissue samples were taken from controls during third molar extraction and from patients before (T1) and 6 months following surgery (T2). In a first step 15 Miroarrays (42525 Microchip: SurePrint G3. Agilent) were applied in 3 controls, 3 prognathic and 3 retrognathic patients (T1, T2) to identify entities to be

differentially expressed between the groups. Relative quantification for 12 genes of interest (developmental, fast and slow myosin heavy chain mRNA (MYH), Calcineurin, COX2, IGF, Myostatin) was performed with real time PCR. Gene regulations were analyzed with the relative expression software tool. Unpaired t test and ANOVA served for statistics.
Microarrays showed decreasing of differentially gene expression between the controls and the patients at T2 e.g. a convergence of gene expression after surgery ($p < 0.05$), more decreased in prognathic than in retrognathic patients. RT-PCR show increase of developmental MYH and switching from slow to fast MYH which signalize improvement of masticatory function. Calcineurin, which is important in the stretching cascade showed in retrognathics a significant higher up regulation. Myostatin expression was fourfold higher in retrognathics. The microarray findings give evidence that function follows form 6 month after surgery in orthognathic patients. But the regeneration process is more advanced in prognathic than in retrognathic patients. The up regulation of stretching factor could delay the regeneration process.

I.P12.

Fluid shear stress stimulates growth factor expression and nitric oxide production by myotubes

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Physical activity increases muscle fiber size by transducing external signals via cell surface receptors into biological responses. Finite-element modeling data suggest that mechanically loaded muscles in vivo do not only experience tensile strain but also shear stress. However it is unknown whether this mechanical loading-induced shear stress affects biological pathways involved in muscle fiber size adaptation to mechanical loading. Our aim was two-fold: (1) to determine whether shear stress affects growth factor expression and nitric oxide (NO) production by myotubes, and (2) to explore the mechanism of this shear stress effect on myotubes in vitro.
C2C12 myotubes were subjected to shear stress resulting from pulsating fluid flow (PFF, 8.8 Pa/s, 1.4 ± 1.4 Pa, 1 Hz, 1 h). Gene expression was analyzed immediately after PFF and after 6 h post-PFF treatment. The glycocalyx was degraded by adding hyaluronidase, and stretch-activated ion channels (SACs) were blocked using $GdCl_3$. Medium NO concentrations were assessed during PFF-treatment.
PFF affected mRNA levels of key-regulators of muscle hypertrophy in myotubes. It increased IGF-I Ea, MGF, VEGF, IL-6, and COX-2 mRNA, but decreased myostatin mRNA expression. The effect of shear stress rate on NO production was dose-dependent. Glycocalyx degradation, and blocking of SACs ablated the shear stress-stimulated NO production.
In conclusion, shear stress activates signaling pathways involved in muscle fiber size adaptation in myotubes, likely via membrane-bound mechanoreceptors. These results strongly support a new concept in muscle biology, i.e. shear stress exerted on myofiber extracellular matrix may play an important role in mechanotransduction in muscle.

I.P13.

Effect of glucosamine-containing supplement on knee pain and locomotor functions in subjects with knee pain

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Objective Oral glucosamine and chondroitin sulfate have been used worldwide for the treatment of knee pain, but their efficacy for locomotor functions, e.g. walking speed has not been elucidated. This clinical study aimed at investigating the potential of a glucosamine-containing supplement for improving locomotor functions.
Methods A randomized double-blind, placebo-controlled study was conducted in 100 Japanese subjects with knee pain (KL grades; 0–II). The subjects were randomly assigned to either supplement containing a daily dose of 1,200 mg of glucosamine hydrochloride, 60 mg of chondroitin sulfate, 90 mg of quercetin glycosides, 10 mg of imidazol peptides and 5 ug of vitamin D (GCQID group, 50 subjects), or placebo (placebo group, 50 subjects), and the intervention was continued for 16 weeks. Efficacy for knee pain was evaluated using VAS. To examine the effect on locomotor functions, normal walking speed and knee extensor strength were measured.
Results VAS Score for knee pain during walking was significantly improved in both groups but the improvement was earlier and larger in GCQID group. Normal walking speed increased at week 8 and 16 in both groups, but there was no significant difference between groups. Knee extensor strength significantly increased at week 8 and 16 only in GCQID group. For subjects with mild-to-severe knee pain at baseline, change in normal walking speed was significantly greater in GCQID group than in placebo group.
Conclusion In subjects with knee pain associated with osteoarthritis, the GCQID supplement might be effective in reducing knee pain and improving locomotor functions.

I.P14.

Changes in the tissue glycogen distribution in a hypermuscular mice model

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The TGF-beta member myostatin is the main negative regulator of skeletal muscle mass. The *Compact* mice carry a naturally occurring 12-bp deletion in the propeptide region of the myostatin precursor, and additional modifiers are involved in determining the full expression of the hypermuscular phenotype. Our aim was the morphological and morphometrical analysis of the *Compact* mice, focusing on the glycogen content and distribution of m. tibialis anterior (TA).
Frozen sections were stained by PAS to visualize glycogen and immunohistochemistry was performed to detect the different muscle fiber types, respectively. Fiber type specific glycogen content was analyzed densitometrically on serial sections. Total glycogen content was measured by spectrophotometry.
The absolute weight of TA muscles significantly increased in *Compact* animals compared to wild type. The whole glycogen content significantly increased in TA (43.6 ± 2.4 vs. $28.7 \pm 0.7681 \times 10^{-2}$mg, $p < 0.01$) of *Compacts*; however, the average glycogen content of the fibers did not change but their number significantly increased. Among the fast fibers, the type IIB fibers contained the most glycogen in both group. The average glycogen concentration in TA samples of *Compacts* was significantly lower (3.72 ± 0.23 vs. 5.52 ± 0.24 mg/g, $p < 0.01$).
The *Compact* mutation influences the glycogen content of the muscle; the decreased glycogen concentration of *Compact* muscle can play a role in the lack of proportionate muscle force increase observed previously.

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I.P15.

Study of influence of hyperthyroidism on calcium regulation of muscle contraction in slow and fast skeletal muscle of rabbit with an in vitro motility assay

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Skeletal muscles contain multiple isoforms of myosin. Thyroxin plays a significant role in expression of myosin genes and it is known that effect of hyperthyroidism is muscle specific. We studied the influence of hyperthyroidism on "pCa-velocity" relationship for myosin from fast (*m. psoas*) and slow (*m. semimembranous*) rabbit skeletal muscles using in vitro motility assay. The isoform composition and carbonyl content of myosin from healthy and hyperthyroid rabbits were identified. Also we measured shortening velocity of permeabilized muscle fibers from the same muscles.

We found that for both muscles of hyperthyroid rabbits: (1) expression of fast isoforms of myosin heavy chains increased; (2) expression of myosin light chain isoforms did not change; (3) carbonyl content of myosin increased two-fold; (4) sliding velocity of regulated thin filaments at maximal calcium decreased about two-fold; (5) the Hill coefficient of "pCa–velocity" relationship increased and calcium sensitivity (taken as pCa_{50}) decreased; (6) maximal shortening velocity of muscle fibers from hyperthyroid rabbits had no significant difference from control.

I.P16.

Cross-bridge attachments in fibre types of human skeletal muscle

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To fulfill various functional needs skeletal muscles consist of different fibre types: slow fibres (type I) containing the myosin heavy chain isoform MHC-I and fast fibres (type II) containing MHC-IIa (type IIA), MHCIId (type IID) or MHCIIb (type IIB).

Previously we provided evidences that slow and fast fibres differ in their types of myosin head attachment. Force-generating attachments of myosin heads were investigated by applying small perturbations of myosin head pulling cycles in stepwise stretch experiments on single skeletal muscle fibres. Rat fast fibres (pure MHC-IIb) exhibited two discrete types of myosin head attachments: a slow type at low levels of Ca^{2+}-activation and an up to 30 times faster type at high levels of activation. In contrast, rat slow fibres displayed only one type of myosin head attachment (slow type) which showed an increase in kinetics from low to maximal level of Ca^{2+}-activation.

Here we extended our investigation to human skeletal muscle fibres. The data obtained so far suggests that slow fibres exhibit only the 'slow-type' of myosin head attachment, like in rat. Its kinetics also increased from low to maximal level of Ca^{2+}-activation. The fast fibres investigated so far contained mainly MHC-IIa; they showed only one type of attachment over the whole range of Ca^{2+}-activation. It was hardly Ca^{2+}-sensitive at a Ca^{2+}-activation level of 50–100 % and about 10 times faster as that of type I fibres.

I.P17.

Age-related changes in neurotrophic factors in human extraocular and limb muscles

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Neurotrophins and neurotrophic factors (NTFs) modulate development, differentiation and maturation of neurons. The survival of developing neurons shortly after their axons reach their peripheral targets is dependent on NTFs. Target-derived NTFs are transported retrogradely from skeletal muscle to spinal cord and ventral horn motor neurons via neuromuscular junctions (NMJs). Thus, failure of muscles to secret sufficient amounts of NTFs might lead to loss of specific neuron populations and lower motor neuron degeneration. Extraocular muscles (EOMs) are a unique muscle allotype and their most striking feature is their differential sensitivity to disease. We investigated the distribution of BDNF, NT3, NT4, GDNF, TrKb and TrkC in EOMs and limb muscles of healthy adults and elderly using immunohistochemistry. NTFs and their receptors were present in various structures in the muscle tissue, including muscle fibers, nerve axons, myotendinous junctions, myonuclei and NMJs. We found alterations of NTFs expression patterns in nerve axons in both EOMs and limb muscle but not at NMJs, indicating different aging effects of NTFs between nerve axons and synapses. Furthermore, the expression of NTFs was down-regulated in nerve axons in EOMs but up-regulated in nerve axons in limb muscles from elderly, which may reflect distinct impact of aging on nerve axons in EOMs versus limb muscles that may be relevant for our understanding of the pathological changes of NTFs distribution in ALS patients.

I.P18.

Differences in contractile properties of single muscle fibres from bodybuilders, wrestlers and untrained controls

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While it has often been observed that the specific tension (P0/CSA) of single fibres is higher in resistance-trained individuals, no studies have yet investigated the impact on specific power (PP kg^{-1}). Therefore, we compared contractile properties of single fibres from bodybuilders (BB), training for muscle bulk, with those from wrestlers (W), training for muscle strength, and non-trained controls (C). Fibres were collected from the vastus lateralis of 7 male BB (30.4 ± 5.1 y), 7 W (25.1 ± 2.7 y) and 9 C (24.3 ± 3.9 y) after giving informed consent. P0/CSA and PP kg^{-1} were determined using maximal isotonic and isometric contractions. Fibre type was determined with SDS-PAGE. Data were analysed with a 2-way ANOVA (Group [BB,WR,CO]) × fibre type [I,IIa,IIa/IIx,IIx]).

P0/CSA was similar in all fibre types. Type I fibres were slower and produced less PP kg^{-1} than type IIa, IIa/IIx and IIx fibres ($p < 0.01$).

PP kg^{-1} of W was 39 % higher than that of C and C had a 42 % higher PP than BB ($p \leq 0.0.01$). PP kg^{-1} of W was 97 % higher than BB ($p < 0.01$). P0/CSA in W was 54 and 30 % higher than BB and C ($p < 0.01$), respectively. The absence of group*fibre type interactions indicates that these differences between groups were similar in all fibre types.
In conclusion, PP kg^{-1} of single muscle fibres appears to be affected by the type of training, which is attributable to differences in P0/CSA between groups. The lower PP kg^{-1} in BB suggest that their type of training has a negative impact on muscle quality, while wrestling training appears to improve muscle quality.

I.P19.

The key markers of proteolytic signaling pathways in m. vastus lateralis among alcohol consumers

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The aim of the study was to analyze the state of the key markers of the proteolytic signaling pathways in m. vastus lateralis among patients with alcohol myopathy with different degree of muscle fibers atrophy. We studied the following groups of patients: (1) control group, (2) group without atrophy of muscle fibers, (3) group with the atrophy of type 2 muscle fibers, (4) patients with atrophy of both type 1 and type 2 muscle fibers. Using western-blotting the content of IRS-1 was established and the expression of E3-ubiquitin-ligases MuRF-1 и MAFbx was assessed by qRT-PCR. In group with the atrophy of type 2 muscle fibers there was a 24 % ($p < 0.05$) decrease of IRS-1 content compared to control. And in patients with the atrophy of type 1 and 2 muscle fibers there was a 39 % decrease of IRS-1 ($p < 0.05$) content. Also we observed a tendency ($p < 0.1$) towards the increase of E3-ubiquitin-ligases expression in a group without muscle atrophy and in a group with the atrophy of type 2 muscle fibers compared to control. Thus in present study for the first time we obtained set of data about the state of crucial markers of proteolytic signaling pathways in m. vastus lateralis among chronic alcohol consumers.

I.P20.

Mathematical representation of fascicular orientation in skeletal muscles and its implications on passive and active deformations

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Skeletal muscles are considered as chemo-mechanical transducers as they convert chemical energy to mechanical energy to perform various tasks. These muscles undergo a range of deformations in day to day activities. The deformations can vary from relatively small, linear to large, nonlinear changes that include bending and torsion. It is well known that the passive mechanical properties of skeletal muscles exhibit transversely isotropic properties. In addition to that when the muscle fibres are activated by the central nervous system forces are generated along the fibre direction. Thus, in order to understand the overall mechanical behaviour of muscles one needs to accurately depict the fibre or fascicular orientation within the muscle. This paper looks at how an orthogonal curvilinear material coordinate system together with three Euler (rotation) angles can be used to define the fibre orientation precisely and its implications on overall deformation mechanics of skeletal muscles. The variation of Euler angles within the muscle was described by tri-linear finite element field. The geometry of the 3-dimensional muscle structure was represented using a finite element model with Hermite family cubic interpolation scheme. Use of Hermite family element ensures the displacement gradient is continuous throughout the muscle domain and hence no physical governing equations are violated. The 3-dimensional geometry of the muscle was created by iteratively fitting an approximate (initial) mesh to the data derived from images.

I.P21.

Molecular signalling response to short duration high intensity/low volume resistance training in human skeletal muscle

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It is known that Resistance Training activates phosphorylation-based intracellular anabolic signalling cascades, but, considering the numerous variables of RT, the differences between training modalities have been poorly investigated. The aim of this study was to analyse Akt, 4EBP1, S6 and AMPK, ACC signalling after a single session of high-intensity resistance training (HIRT) technique and after a traditional resistance training (TRT).
HIRT consisted in 2 sets of 6/2/2 reps with incomplete rest between (20^{-1}) sets, while TRT consisted of 4 sets × 15 reps with 1′15″ of rest between sets. 12 healthy young subjects performed each protocol in two different moments and with different legs. Biopsy samples were collected from the vastus lateralis with a fine needle 1 week before training (pre) sessions, immediately after (T0), 6 h after (T6) and 24 h after (T24) training. Akt, 4EBP1, S6, AMPK and ACC phosphorylation was determined by Western blotting.
No significant difference was found at any of time point after exercise in AKT and 4EBP1 phosphorylation. In contrast, there was a significant increase in S6 phosphorylation at T6 both in HIRT and TRT. S6-P remained at higher level even at T24.
Our findings suggest that resistance training technique requiring a lower time commitment is equally effective to induce an increase in S6-P. The increase of the phosphorylated form of S6 without a concomitant increase of Akt-P could be explained with the selected biopsy timing (after 6 h in our experiment, after 1or 2 h in other studies) or with an Akt-independent S6 phoshorylation.

I.P22.

The effect of calpain inhibition on signaling pathways in m.soleus under hindlimb unloading of rats

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We hypothesized that calpaines can serve an initial link which launch ES further protein ubiquitination under gravitational unloading of muscle. The aim of our study was to evaluate the role of calpaines in the regulation of muscle protein metabolism during hindlimb unloading: the work of ubiquitin–proteasome as well as protein synthesis (Akt-mTOR-S6K and MAPK/Erk) pathways. For this reason we inhibited μ-calpaines in m.soleus of rats by PD150606 administration during 3-day hindlimb unloading (group UPD, $n = 7$). Gr. U was suspended without treatment, gr.C served as Control (C, $n = 7$). We found that μ-calpain mRNA level did not increase and soleus atrophy was prevented in UPD group (in contrast to U group).

mRNA E3 ligase atrogine-1(but not MuRF-1) and conjugated ubiquitin levels did not increase in this group either (as opposed to the U rats). The pFOXO3 content was equal in control and UPD groups as well as the level of eEF2 k mRNA expression (in contrast to U group, $p < 0.05$). At the same time nNOS and HSP70 mRNA expression in U soleus was reduced (in comparison to C group, $p < 0.05$). Conclusion: the raise of μ-calpain level under hindlimb suspension leads to the increase of proteins ubiquitination and atrogine-1 level (which represented the ubiquitin–proteasome pathway).The change of μ-calpain level expression is associated with the regulation of protein synthesis, which is controlled not only by Akt-mTOR-S6K signaling pathway, but also by other factors regulating it at the level of elongation.

I.P23.

Effects of endurance training, resistance training and neuromuscular electrical stimulation (NMES) on myonuclei and satellite cells density

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It is still debated whether myonuclear addition is required for muscle hypertrophy and whether satellite cell activation accompanies muscle training. In this study we aimed to assess the impact of resistance and endurance training on myonuclear density and myonuclear domain size in human muscle fibers.
Muscle biopsy samples were collected from vastus lateralis muscle of 10 young healthy subjects (age 22–29 y) before and after 2 months of conventional resistance training, from 7 young adult subjects (age 30–54 y) before and after 40 days mountain trekking and from one subject before and after 6 weeks of neuromuscular electrical stimulation (NMES). The effects of training were confirmed by measuring muscle mass and strength for resistance training and NMES and by measuring maximum oxygen consumption for endurance training.In single muscle fibers manually dissected from biopsy samples, fixed with paraformaldehyde and permeabilized with Triton, nuclei were visualized with Hoechst and satellite cells stained with anti Pax-7 monoclonal antibody.
The myonuclear density was increased by all three training protocols, indicating not only an activation of satellite cells and myonuclear incorporation but also that the addition of new myonuclei was higher than required to keep constant the nuclear domain volume. Interestingly, the satellite cells, expressed as percentage of total nuclei number, remained unchanged or even decreased at the end of the training period. These findings strongly suggest that myonuclear addition, likely via satellite cells recruitment, is required to achieve myofiber hypertrophy after resistance training and NMES and also for adaptation to endurance training in humans.

I.P24.

Alp/enigma family proteins cooperate in Z-disc formation and myofibril assembly

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The *Drosophila* Alp/Enigma family protein Zasp52 localizes to myotendinous junctions and Z-discs. It is required for terminal muscle differentiation and muscle attachment. Its vertebrate ortholog ZASP/Cypher also localizes to Z-discs, interacts with α-actinin through its PDZ domain, and is involved in Z-disc maintenance. Human mutations in ZASP cause myopathies and cardiomyopathies. Here we show that *Drosophila* Zasp52 is one of the earliest markers of Z-disc assembly, and we use a Zasp52-GFP fusion to document myofibril assembly by live imaging. We demonstrate that Zasp52 is required for adult Z-disc stability and pupal myofibril assembly. In addition, we show that two closely related proteins, Zasp66 and the newly identified Zasp67, are also required for adult Z-disc stability and are participating with Zasp52 in Z-disc assembly resulting in more severe, synergistic myofibril defects in double mutants. Zasp52 and Zasp66 directly bind to α-actinin, and they can also form a ternary complex. Our results indicate that Alp/Enigma family members cooperate in Z-disc assembly and myofibril formation; and we propose, based on sequence analysis, a novel class of PDZ domain likely involved in α-actinin binding.

I.P25.

Signaling effects in reloaded muscle: eccentric-like events

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D. Riley found that during the first hours and days of reloading after exposure to unloading the ultrastructural alterations in postural muscle resembled the eccentric-induced damages. The present study was purposed to evaluate the state of muscle cytoskeletal proteins and key markers of the signaling pathways at the different stages of muscle reloading after 14-day rat hindlimb unloading. We revealed that the content of desmin and α-actinin dramatically decreased in rat soleus at the early stage of reloading (3 days) and then increased sufficiently by the 7th day of reloading which looked like the eccentric-induced changes. It is known that at the 3rd day of reloading the content of the phosphorylated p70S6K is significantly increased. We found the sufficient reduction of thep70S6K phosphorylation level after 7 and 14 days of reloading. The phosphorylation of the ribosomal kinase p90RSK was found to be decreased (as compared to the unloading level) after 7 days of reloading. The mRNA contents of the key E3-ubiquitin ligases MuRF-1 and MAFbx/atrogin-1were increased after 3 days of reloading as compared to the control level, and reduced by the 7th day of reloading. Thus the signaling events during reloading as well as the cytoskeletal protein degradation are supposed to be the consequences of the eccentric-like behavior of rat soleus at the early stages of reloading. The study was supported by the Physiological Program of the Presidium of the Russian Academy of Sciences.

I.P26.

cAMP/PKA signaling activation induces foxo1 phosphorylation and suppresses ubiquitin–proteasome system in skeletal muscle

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It is well established that catecholamines inhibit skeletal muscle protein degradation, but the molecular underlying mechanism remains unclear. This study tested the hypothesis that the ubiquitin–proteasome system (UPS), which is the major proteolytic system in skeletal muscle, could be regulated by catecholamines through the cAMP/

PKA signaling. The in vitro effects of norepinephrine (NE), forskolin (FSK; a adenylyl cyclase activator) and 6-Bnz-cAMPS (a selective PKA activator) on expression of atrophy-related genes (Atrogin-1 and MuRF1), and on cAMP/PKA and Akt/Foxo signaling were examined in either C2C12 cells or extensor digitorum longus (EDL) muscles. Additionally, rates of proteolysis in isolated EDL muscles from rats were determined by measuring the tyrosine release in the incubation medium. In EDL muscles, the addition of NE (10–4 M) increased muscle cAMP levels (115 %), the phosphorylation of PKA substrates (3.1-fold), and the phosphorylation of Foxo1 (~6-fold), but did not alter the phosphorylation of Akt. Furthermore, NE reduced the basal UPS activity (21 %), and the gene expression of Atrogin-1 (64 %) and MuRF-1 (43 %). The PKA activator 6-Bnz-cAMPS (500 μM) increased the phosphorylation levels of CREB and Foxo1 and reduced UPS activity (~45 %). In cultured C2C12 myoblast, activation of cAMP/PKA signaling induced by FSK (10 μM) increased the phosphorylation levels of CREB and Foxo1, but not Akt. Moreover, 6-Bnz-cAMPS reduced the mRNA levels of Atrogin-1 in cultured C2C12 myotubes. These data suggest that activation of cAMP/PKA signaling induced by NE inhibits UPS activity through a novel mechanism that involves phosphorylation and inhibition of Foxo1, independently of Akt.

I.P27.

Effects of hypo/hypergravity variations on mice soleus plasticity

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Our aim was to understand how gravity determines the properties of muscle function in mammals: for instance, to understand if there is a continuum, i.e. a connection between the direction (<0G or >0G) i.e. the magnitude of gravity vector (0G ↔ 4G) and the muscular effects measured (has hypergravity an opposite effect to microgravity? is the effect of hypergravity (HG) "dose-dependent"?)
This study was conducted on adult mice, divided in control, microgravity (21 days, 0G); and hypergravity (21 days, chronic centrifugation) groups. The experiments focused on the morphological (mice and muscle weights), contractile in situ muscle properties, and biochemical studies: soleus phenotype by both myosin heavy chains (MHC) immunohistochemistry on muscle sections and determination of the expression of MHC isoforms by 1D-electrophoresis. A decrease in muscle wet weight/body weight was observed in 0G muscles, whereas HG induced an increase in this parameter only at 3 and 4G. The 0G muscle evolved towards a faster type (neo-expression of myosin IIX), while 3G muscles presented a slower phenotype. The results were assessed by contractile behaviour.
Moreover, we addressed the changes regulation in muscle mass (atrophy in 0G and hypertrophy in HG conditions) and analyzed the activation level of specific cellular signalling pathways, anabolic PI3K-AKT-mTOR markers: AKT, mTOR (mammalian Target Of Rapamycin), p70S6K and 4E-BP1, and catabolic PI3K-AKT-FOXO markers: FOXO1, MuRF1 and Atrogin-1. We hypothesize that muscle mass in 0G and HG conditions is differentially regulating by these signalling pathways.
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I.P28.

Divergent in vivo muscle contractions cause distinct skeletal muscle focal adhesion/costamere adaptations and related downstream signaling pathways

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Mechanotransduction of physical forces into biochemical signals enables skeletal muscles (SkM) to adapt to exercise, while the impact of different muscle contraction modes remains unclear. Focal adhesion (FA) and costamere (CSTM) protein assemblies are important structures assuring proper SkM adaptations to loading. Only little knowledge exists, however, on effects of in vivo loading on FAs/CSTMs, although this question is of high relevance to understand regulatory potentials of loading and contraction modes on SkM. We hypothesized that FAs/CSTMs and downstream signaling cascades are regulated loading and contractions mode-dependently.
Sprague–Dawley rats were assigned to the following groups: sedentary (CON, n = 7), 6-weeks lasting concentric level running (Level, n = 7, 5 days/week, 1 h/day, 20 m/min, 0° decline) or 6-weeks lasting eccentric downhill running (Dh, n = 7, see Level, but −20° decline). Quadriceps femoris (QUF) and gastrocnemius (GAS) muscles were investigated. RT-PCR/WB analyses were performed to investigate FAs/CSTMs in both SkM after training interventions.
FAs/CSTMs were jointly regulated in dependence on running/contraction mode and SkM type. Interestingly, Level increased all targets only in QUF, whereas Downhill had no effects. Conversely, Downhill increased all targets only in GAS and no effect was observed in QUF. Downstream signaling cascades, e.g. pp38, pERK, and pAkt were activated in the same manner.
Our data are the first to demonstrate that the type of in vivo muscle contraction has important, but divergent effects on FAs/CSTMs in a contraction- and SkM type-dependent manner. As FAs/CSTMs are involved in a variety of SkM diseases, these data are important to understand SkM adaptations and to evaluate exercise models as therapeutic interventions in SkM diseases.

I.P29.

Regulation of skeletal muscle micrornas in young and old adults following acute resistance exercise

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Introduction Sarcopenia is the age-related loss of muscle mass and function. The onset and progression of sarcopenia is associated with attenuated activation of Akt/mTOR signalling and muscle protein synthesis (MPS), in response to anabolic stimuli such as resistance exercise. MicroRNAs (miRNAs), small regulatory molecules, can influence muscle growth and regeneration. Regulation of miRNAs following resistance exercise, that influence Akt/mTOR signalling and MPS, are poorly understood. This study investigated changes in miRNA expression following an acute bout of resistance exercise in young and old subjects, with a focus on miRNA species predicted to target Akt/mTOR signalling and MPS.
Methods Ten young (18–30 years) and 7 older (60–75 years) males completed knee extensor exercise (3 × 14 repetitions) at 60 % of maximal voluntary contraction. Muscle biopsies were collected before and 2 h after exercise. Akt and mTOR protein levels were

measured by Western blot. miRNA expression was measured using TaqMan® microRNA arrays and analysed bioinformatically (miRWalk) for their association with the Akt/mTOR pathway and MPS.
Results Following resistance exercise, p-Akt increased in young (n = 8), but not in old subjects (n = 4). miR99b, −100, −149 and −370 were decreased only in the young subjects, while miR-355-3p was decreased and miR-99a, −124 and −499-5p were increased only in the old subjects.
Conclusion This study identified 8 miRNAs potentially regulating Akt/mTOR signalling following resistance exercise in young and old people. Modulating their expression levels in human primary muscle cells will provide further insight into their causal role in regulating Akt/mTOR signalling and MPS.

I.P30.

Suppression of mTORC1 activation in acid-α-glucosidase deficient disease is ameliorated by leucine supplementation

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Deficiency in acid-α-glucosidase (GAA) results in a debilitating skeletal muscle wasting in Pompe disease characterized by the accumulation of glycogen and autophagic vesicles and by elevated lysosome pH. Suppression of mTORC1 activation was demonstrated in skeletal muscle of Pompe mice, GAA-knockdown C2C12 myoblasts and GAA deficiency in human skin fibroblasts of infantile Pompe patients. Leucine supplementation partially restored mTORC1 activation toward control stimulatory levels, whereas the more permeable L-leucyl–L-leucine methyl ester was more effective in GAA deficient cultured cells. In vivo, Chronic leucine feeding also partially rescues mTORC1 activation in Pompe mice and protected Pompe mice from developing kyphosis, decreased muscle mass, spontaneous activity and running capacity while reconstructing balance of muscle protein synthesis and breakdown and reducing glycogen accumulation. Meanwhile, due to improving muscle function, glucose metabolism becomes normal in Pompe mice. Together, these data demonstrate that GAA deficiency results in reduced mTORC1 activation that is partly responsible for the skeletal muscle wasting phenotype. Moreover, chronic mTORC1 stimulation by dietary leucine supplementation was able to prevent some of the detrimental skeletal muscle dysfunction that occurs in the Pompe disease mouse model.

POSTER SESSION II Muscle regeneration

II.P1.

Klf5 modulates proliferation and differentiation of myoblasts

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Development of skeletal muscle and satellite cell activation are regulated by myogenic regulatory factors. In addition to these, several other transcription factors are identified to play roles in the control of skeletal muscle maintenance. For the first time we have identified that Klf5, a ubiquitous zinc finger transcription factor, is upregulated in the course of myoblast differentiation in vitro and muscle regeneration in vivo. Both primary myoblasts and myoblast cell lines induce Klf5 expression up to tenfolds in the course of myotube formation. Similarly, following acute myofiber necrosis, Klf5 is upregulated concomitantly with the formation of young myofibers and returns to baseline levels upon restoration of the tissue architecture. Likewise, when chronic degenerative conditions such as inherited myopathies and the diaphragm of the mdx mouse are investigated, Klf5 expression is also observed to be induced. In light of these observations we show that Klf5 is sumoylated and translocated to the nucleus with differentiation in vitro and regeneration in vivo. Conditional silencing of Klf5 interferes with myoblast proliferation, fusion and myotube maturation. Klf5 silencing strikingly induced the proliferation rate of myoblasts, inhibited fusion as well as myotube maturation. On the other hand, overexpression of Klf5 induces cell cycle arrest. This data provide evidence that Klf5 is a novel modulator of skeletal muscle regeneration and differentiation exerting an anti-mitotic effect on committed myoblasts. Our further efforts are focused on identification of its genomic targets in the muscle tissue.

II.P2.

SKNAC: a multi-functional regulator of myogenesis and skeletal muscle regeneration

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skNAC (skeletal and heart muscle specific variant of nascent polypeptide associated complex) is a skeletal and heart muscle-specific splice variant of the translational coactivator aNAC. The *skNAC* gene is strongly expressed during myogenic differentiation and muscle regeneration. Others and we could also recently show that skNAC plays a critical role in sarcomerogenesis. The respective mechanism, however, is largely unknown. While testing the expression of a broad range of genes known to be involved in myofibril organization we found that skNAC regulates *calpain* expression. Calpains are a family of cytosolic, Ca^{2+}-dependent cysteine proteases, consisting of the ubiquitous calpains 1 and 2, and calpain 3, which is mainly present in skeletal muscle. At least calpain 3 is known to be a central regulator of myofibril assembly, turnover, and maintenance, which is impressively illustrated by the fact that mutations in this gene cause the human disease limb girdle muscular dystrophy type 2A. We could demonstrate that inhibition of *skNAC* expression in skeletal muscle cells leads to enhanced, and overexpression of the *skNAC* gene to repressed, expression of calpain 1 and—to a lesser extent—calpain 3, resulting in altered enzymatic activities. In skNAC siRNA-treated cells, enhanced calpain activity was associated with increased migration rates, as well as with perturbed sarcomere architecture. Treatment of skNAC knockdown cells with the calpain inhibitor ALLN reverted both the positive effect on myoblast migration and the negative effect on sarcomere architecture. Taken together, our data suggest that skNAC controls myoblast migration and sarcomere architecture in a calpain-dependent manner.

II.P3.

Do humans and flies share the same molecular mechanisms in regulating muscle cell fusion events?

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Introduction Somatic cell fusion is critical for skeletal muscle growth and regeneration following injury such as exercise-induced damage. The occurrence of somatic cell fusion in skeletal muscle offers a potential mechanism for delivery of a desired gene to cure genetic myopathies via cell therapy. Our understanding of the molecular mechanisms regulating muscle cell fusion in humans is currently rudimentary. Significant progress has been made in identifying genes responsible for muscle cell fusion events during embryogenesis in Drosophila Melanogaster. Two type 1 transmembrane proteins, members of the immunoglobulin superfamily termed Kin of Irre (Kirre) and roughest (Rst), are essential for muscle cell fusion in Drosophila. It is currently unknown whether a human homolog of Kirre and Rst, Kirrel is present in adult human skeletal muscle.
Aim To assess for the possible presence of the evolutionary conserved Kirrel gene in adult human skeletal muscle biopsy samples.
Methods and results The presence of at least two Kirrel mRNA transcripts in adult human skeletal muscle was demonstrated. One of these is unreported in the published literature. Furthermore SDS PAGE and immunodetection data suggest that the expression levels of the novel Kirrel protein isoform alters during the recovery period after a high-intensity downhill running intervention.
Conclusion Our results justify the need for more thorough investigation of similarities between fly and humans in the molecular mechanisms which regulate muscle cell fusion events. Further, relatively minor muscle damage in otherwise healthy individuals is a model that is sufficient to test changes in potential fusion gene expression.

II.P4.

Calcium/calmodulin-dependent kinase preserves contractility of injured skeletal muscle

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Injury-induced rises in myocellular calcium concentration are a confounding variable of experimental paradigms assessing muscle's response to external forms of stress. We have tested the hypothesis that elevated expression of a main intracellular receptor of calcium, Calcium/Calmodulin-dependent kinase (CaMKII) isoform is implicated in the regenerative/phenotypic response of skeletal muscle to injury. Electro-injury (3 trains of 80 pulses of 100 microsecond duration, each at 100 mA applied at two sites) doubled the content of δA and δD/γB CaMKII isoforms in slow type soleus muscle and prolonged the twitch time-to-peak force ($p = 0.05$) and half-relaxation time ($p = 0.03$) with a concomitant drop in tetanic force ($p = 0.002$). Transcript profiling of ubiquitous calcium binding proteins identified threefold elevated expression of the calcium buffering protein annexin V after electro-injury and a twofold reduced expression of the major calcium pump resetting myocellular calcium, SERCA2 (statistical analysis for microarrays, q value <0.05). Transfection with expression constructs for native alpha and beta CaMKII isoforms normalised twitch time-to-peak force and half-relaxation time in electro-injured soleus and fast type gastrocnemius medialis muscle. The improvement in soleus muscle was related to elevated content of annexin V (threefold, $p = 0.03$) and SERCA2 (1.2-fold, $p = 0.001$) protein and a 1.2-fold increased content of fast a/x type myosin heavy chains ($p = 0.04$). The findings are compatible with the view that CaMKII expression is upstream of a compensatory response of calcium handling which governs contractility in injured muscle and motivates CaMKII as target for gene therapy of muscle damage.

II.P5.

HGF receptor agonists protect cardiomyocytes from $CoCl_2$-induced cell injury

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Met, the tyrosine kinase receptor for HGF, controls a genetic program including protection from apoptosis and cell survival. This program is known to play a key role in embryo development, organ regeneration and cancer. Here we show that H9c2 cardiomyoblasts express the Met receptor and respond to HGF by activating the downstream signaling pathways, including mTOR. A superimposable response was elicited by the agonist monoclonal antibodies. $CoCl_2$-induced hypoxia promotes HIF-1α stabilization and transcriptional induction of itself and hypoxic gene targets. The HIF-1α activation leads to reduction of cell viability and apoptotic damage. Furthermore, the stimulation of hypoxic pathway induced the pro-autophagic Redd1 and Bnip3 mRNAs and AMPK phosphorylation, which lead to inhibition of mTOR pathway and excessive autophagy. Importantly, the presence of HGF or Met agonist antibodies protected the cardiomyoblasts from reduction of cell viability, excessive autophagy and cell death. This protective effect was mediated by the activation of mTOR pathway. These data show that Met activation protect cardiac cells from ischemic injury by dual mechanisms, including apoptosis protection and inhibition of autophagy, providing a promising therapeutic tool for ischemic injury.

II.P6.

Role of macrophagic HIFs in the resolution of inflammation during skeletal muscle regeneration

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The critical role of macrophages (MPs) and the corresponding inflammatory response associated to the muscle repair process has been recently highlighted. Recent studies have demonstrated that HIF-1α could play a key role in inflammation. One could thereby expect an impaired/delayed muscle regeneration process as a result of HIF deletion in MPs. We addressed in vivo and in vitro the role of HIFs in MPs in the resolution of inflammation during skeletal muscle regeneration.
Acute muscle injury was induced by the *i.m.* injection of cardiotoxin (CTX) in the *tibialis anterior* muscle. Experiments were performed in transgenic mice deleted for HIF-1α or for HIF-2α in MPs (i.e., LysM-Cre;HIF-$1\alpha^{fl/fl}$ or LysM-Cre;HIF-$2\alpha^{fl/fl}$) and in wild-type (WT) mice before and 1, 2, 3, 7 and 21 days after CTX injection. Magnetic resonance imaging was performed in vivo on a 11.75 T MRI system. Diffusion Tensor Imaging (DTI) metrics and T_2 values were measured. In vitro and ex vivo setups were also used to analyze the role of macrophagic HIFs.

Both DTI metrics and T_2 values were significantly modified by CTX injection but these changes were similar for both WT and transgenic mice. The capability of HIF-1$\alpha^{-/-}$ and HIF-2$\alpha^{-/-}$ MPs to switch their phenotype from a pro to an anti-inflammatory phenotype were identical to WT MPs.
We found that neither HIF-1α nor HIF-2α are involved in the muscle regeneration process resulting from an acute injury, suggesting that the role of macrophagic HIFs in inflammation may vary according to the type of inflammation (i.e., sterile vs. infection).

II.P9.

SFRP2 designates skeletal muscle fibrosis in immobilized muscle

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Chronic muscle degeneration is the end-point of congenital muscle dystrophies with manifestations of fiber atrophy, endomysial fibrosis and fatty infiltration. While genetic models do not completely model this picture, immobilization in rat by the tenotomy of the Achilles tendon lead to time-course atrophy associated with endomysial fibrosis and fatty infiltration.
We have conducted a microarray analysis in a cohort study of the tenotomy immobilized rat muscles and observed early upregulation of a battery of genes associated with tissue remodeling and fibrosis. This approach showed early activation of the previously defined atrophy pathways in tenotomy. As well as the upregulation of numerous isoforms of collagens, extracellular matrix proteins, serine proteases and matrix metalloproteinases which imply that the tissue remodeling and fibrosis were initiated at an early stage in muscle immobilization. One key observation is the concomitant early expression of secreted frizzled related protein 2 (SFRP2). SFRP2 expression is strongly localized to the slow-twitch (type II) muscle fibers in tenotomy degeneration. SFRP2 is known as a regulator of Wnt signaling and implicated in regulation of cell fate and differentiation. Novel but controversial roles are attributed in the cardiac muscle; either as an inhibitor of Bmp1 or as an enhancer of procollagenase. While there is no regenerative response in tenotomy, it is highly likely that SFRP2 is acting as a modulator of endomysial fibrosis and is a potential therapeutic target in avoiding fibrosis.

II.P10.

Overexpression of hemeoxygenase-1 in murine myoblasts stimulates blood flow recovery after ischemia

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Hemeoxygenase-1 (HO-1) is a heme degrading enzyme, proved to be cytoprotective and proangiogenic in many different cell types. We have shown recently, that HO-1 exerts also a potent effect on muscle progenitor cells (murine myoblasts C2C12 and primary muscle satellite cells), inducing their viability and proliferation, while diminishing their differentiation. HO-1 was also shown to be decreased in diabetic conditioned what may lead to disturbed angiogenesis and wound healing. Therefore, the aim of this study was to examine the paracrine effect of conditioned media from C2C12 overexpressing cells on blood recovery in diabetic mice.
Conditioned media collected from differentiating C2C12 cells (control and HO-1 overexpressing) were injected into ischemic hind limbs of diabetic mice. Analysis of blood flow by means of laser Doppler system revealed accelerated recovery in animals treated with media from HO-1 overexpressing cells, while subsequent investigation of vessels density in injected muscle confirmed increased angiogenesis. The effect is probably mediated by SDF-1α proangiogenic factor, as its expression is significantly elevated in HO-1 overexpressing cells. Importantly, such a treatment did not induce neoplastic growth. Contrarily, when C2C12 cells with HO-1 overexpression were directly injected into *gastrocnemius muscle* of NOD/SCID mice remained in undifferentiated, proliferating state and created sarcomas with decreased expression of major differentiation regulatory factor—MyoD.
In conclusion, accellular strategy of paracrine stimulation of angiogenesis may be a safer treatment exploiting protective and proangiogenic properties of HO-1 in diabetic conditions.

II.P11.

Murine muscle engineered from dermal precursors: an in vitro model for skeletal muscle generation, degeneration and fatty infiltration

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Skeletal muscle can be engineered by converting dermal precursors into muscle progenitors and differentiated myocytes. However, the efficiency of muscle development remains relatively low and it is currently unclear if this is due to poor characterization of the myogenic precursors, the protocols used for cell differentiation, or a combination of both. In this study, we characterized myogenic precursors present in murine dermospheres, and evaluated mature myotubes grown in a novel 3D culture system. After 5–7 days of differentiation we observed isolated, twitching myotubes followed by spontaneous contractions of the entire tissue-engineered muscle construct on extracellular matrix (ECM). In vitro engineered myofibers expressed canonical muscle markers and exhibited skeletal (not cardiac) muscle ultrastructure, with numerous striations and presence of aligned, enlarged mitochondria, intertwined with sarcoplasmic reticula (SR). Engineered myofibers exhibited Na^+- and Ca^{2+}-dependent inward currents upon acetylcholine (ACh) stimulation and tetrodotoxin-sensitive spontaneous action potentials. Moreover, ACh,

nicotine and caffeine elicited cytosolic Ca^{2+}-transients; fiber contraction coupled to these Ca^{2+}-transients suggest Ca^{2+} entry is activating calcium-induced calcium release from the SR. Blockade by d-tubocurarine of ACh-elicited inward currents and Ca^{2+}-transients suggests nicotinic receptor involvement. Interestingly, after 1-month engineered muscle constructs showed progressive degradation of the myofibers concomitant with fatty infiltration, paralleling the natural course of muscular degeneration. We conclude that mature myofibers may be differentiated on ECM from myogenic precursor cells present in murine dermospheres, in an in vitro system that mimics some characteristics found in aging and muscular degeneration.

II.P12.

Pax7 positive/satellite cells in human extraocular muscles

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The extraocular muscles (EOMS) have been proposed to have higher regenerative capacity than other muscles. We used simultaneous evaluation of two satellite cell markers (Pax7, NCAM)), the position of the identified cell in relation to the myofiber basal lamina and the presence of a nucleus stained by DAPI to study the muscle stem precursors in human EOMS. Signs of satellite cell (SC) activation were analysed with MyoD, myogenin or Ki-67 co-stained for laminin and DAPI.
Many Pax7 positive cells were identified in positions other than the classical SC niche, either surrounded by a basal lamina or not. Some Pax7 positive cells were located close to vessels. Therefore, the proportion of all Pax7 positive cells per myofiber cross-section (Pax7/F) was determined. The proportions of Pax7/F were similar in adult and aged individuals: mean 0.028 ± 0.006 in the middle and 0.031 ± 0.006 in the posterior part of the EOMs but higher in the anterior portion (0.076 ± 0.026 Pax7/F). Staining for both MyoD and myogenin was negative. Co-staining for Pax7 and Ki-67 was not observed.
In summary, the proportion of Pax7 positive cells differed along the length of the EOMs. In the midbelly and posterior parts the proportion of Pax7 positive cells was lower than previously reported in human EOMs (0.07 Pax7/F) and limb muscles (0.07–0.19 SC/F) and similar to child limb muscle (0.032 SC/F). These data are in line with our previous results from limb muscles showing a positive correlation between the number of SCs per myofiber and the mean myofiber cross-section area.

II.P13.

Effects of 1,25-dihydroxyvitamin D on proliferation and differentiation of C2C12 myoblasts

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Vitamin D serum levels are positively associated with muscle strength, suggesting its role in regulation of muscle fiber size. The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), can act through binding to the vitamin D receptor (VDR) which is expressed in muscle. However, little is known about the mechanisms via which vitamin D regulates muscle fiber size. Here we investigated whether $1,25(OH)_2D$ affects proliferation and differentiation of myoblasts and whether it stimulates hypertrophy of myotubes.
Mouse C2C12 myoblasts were cultured in growth medium for up to 1 or 4 days or in differentiation medium for 1 or 3 days in the presence of 0 or 100 nM $1,25(OH)_2D$. mRNA expression levels of VDR, CYP24, ki67, MyOD and myogenin were quantified using RT-qPCR. C2C12 myoblasts were nearly confluent after 4 days of culture in growth medium without $1,25(OH)_2D$. Myoblasts cultured in growth medium showed an increase in myogenin mRNA levels at day 4 compared to that at day 1 which was reduced for 63 % by $1,25(OH)_2D$. During proliferation, $1,25(OH)_2D$ did not affect mRNA levels of ki67 and MyOD.
During culture in differentiation medium, myogenin mRNA levels at day 3 were increased compared to that at day 1, but no effect of $1,25(OH)_2D$ was present. Myotube size as well as MyOD mRNA levels were also not affected by $1,25(OH)_2D$.
The metabolite $1,25(OH)_2D$ increased mRNA levels of both VDR and CYP24 in proliferating as well as differentiating cells which indicates that $1,25(OH)_2D$ regulates its own activity in muscle cells.
These results suggest that $1,25(OH)_2D$ may have an effect in the regulation of muscle fiber size by its role in activation or differentiation of myoblasts. These effects are either co-determined by other serum factors or by signalling molecules particularly available at distinct states of myoblast activation/differentiation.

II.P14.

Significant myogenin expression is not required to synthesise small muscle fibres after in vivo injury in transgenic mice

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Myogenin is one of the basic helix-loop-helix myogenic regulatory factors and is considered to be mainly responsible for promoting myoblast differentiation, myotube and muscle fibre formation. The purpose of this study was to investigate both in vivo and in vitro, how myogenin is implicated in skeletal muscle repair in postnatal life. We used $Myog^{flox/flox}ER^{+/-}$ (M) and wild type (WT) mice (breeding pairs a gift from WH Klein and E Meadows). 3 weeks after M were injected with Tamoxifen, mice were anaesthetized and subjected to a contusion injury of the *gastrocnemius*. WT-Injured mice had fully regenerated by 18 days, whereas M-Injured had grossly abnormal muscle, although small new fibres were present. Next, we isolated primary satellite cells from uninjured muscle. In vitro, two sub-populations of cells were identified: Immature activated satellite cells (SC): $pax7^+$, $MyoD^+$, $Myog^-$; Myogenic precursor cells (mpc): $pax7^-$, $MyoD^-$, $Myog^+$). For the transgenic mice the major population isolated was SC, while for WT mainly mpc. After 4 days in differentiation media, SC and mpc from both groups formed myotubes, although fewer nuclei were present in myogenin-deficient myotubes. These data suggest that less differentiated progenitor cells play a main role in repair by generating new small fibres, while myogenin is required for expression of specific proteins that allow for fusion of more differentiated myoblasts in vitro and for fusion of activated satellite cells to regenerating and new fibres in vivo.

II.P15.

MS1/stars—a novel cardiac stress response transcription factor?

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MS1 (myocyte stress 1), also known as STARS (striated muscle activator of rho signalling) or ABRA (actin binding rho activating protein) was discovered as a protein that is rapidly overexpressed in cardiomyocytes in aortic banding experiments. MS1 is assumed to regulate translocation of MRTFs to the nucleus via polymerisation of G- to F-actin in conjunction with rho. MRTFs released from G-actin can act as cofactors to SRF to drive transcription of muscle specific genes. To better understand this mechanism we performed an extensive dissection of the domain organisation of the protein and determined the structure of its C-terminal domain, the only folded part of the protein. Unexpectedly, its fold is very similar to winged helix-loop-helix DNA binding domains commonly found in transcription factors. As a result it appeared possible that MS1 might play a more direct role in transcription regulation by translocating to the nucleus and binding to DNA itself. So far, however, MS1 has not been seen in the nucleus and the homologous protein to its C-terminus, COSTARS, does not possess DNA binding properties.
We report here that MS1 in able to bind to DNA, that DNA binding is sequence specific with good binding affinity in vitro, that DNA sequence recognition does involve the canonical DNA recognition features of winged helix-loop-helix domains and that MS1 translocates to the nucleus in a highly cell type and development stage specific manner.

II.P16.

Functional analysis of αB-crystallin's ortholog in muscles development and sarcomers stabilisation in *Drosophila melanogaster*

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Drosophila CG 4533 (l(2)efl) gene, the ortholog of αB-crystallin involved in human Desminopathies codes for a small heat shock protein (sHSP). In 3rd instar larva muscles L(2)efl is located around nuclei, on Z-disc and M-line extensions, linking sarcomeres with muscle membrane. At these locations L(2)efl co-localizes with a potential Drosophila intermediate filament (IF) protein detected by antibodies directed against mouse Vimentin. Using co-IP approaches we confirm that L(2)efl interacts with Vimentin-like and identify corresponding candidate proteins. Under attenuation of l(2)efl expression the Vimentin-like protein is mis-localized and a number of perturbations occur in muscles. We observe muscle splitting, altered sarcomeric organization, muscle size, reduced number of nuclei and incorrect muscle attachment. Ultrastructural EM analyses also reveal abnormal swelling mitochondria with broken cristae.
In human, R120G substitution in αB-crystallin leads to the loss of its IF chaperone activity and induces aggregations of Desmin in muscles. We took an advantage of conservation of αB-crystallin to test whether in Drosophila mutated L(2)efl displays similar properties. We found that mesoderm-targeted expression of either mutated L(2)eflR120G or human αB-crystallinR120G lead to the formation of intracellular aggregates containing Vimentin-like and in consequence to muscle weakness. Those symptoms are characteristic for patients with DRM. Thus, the mis-arrangement of intermediate filaments observed in muscles with attenuated l(2)efl expression or expressing αB-crystallinR120G appear reminiscent of defects in human Desminopathies suggesting that Drosophila represents a suitable model system to study DRM.

POSTER SESSION III Cardiac muscle disease—right and left

III.P1.

The role of the pulmonary veins and left atrium in the remodelled human atria during atrial fibrillation

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Atrial fibrillation (AF) is the commonest sustained cardiac arrhythmia which can predispose to heart failure, stroke and even sudden death. The region of the pulmonary veins (PVs) in the left atrium (LA) is associated with the dominant frequencies during AF, which may indicate the presence of driving rotor waves. However, the underlying mechanisms by which the PVs are predisposed to the presence of re-entrant activity in the healthy and remodelled atria are unclear. Elucidation of such mechanisms may help in the prevention and treatment of AF. We apply and update a model of the 3D human atria for such investigation: the family of regional cell models is extended, into which four distinct models of AF-induced remodelling are incorporated. Cell models are then mapped onto a fully segmented anatomical model. Vulnerability windows to conduction block and re-entry are investigated at the major heterogeneous junctions of the PVs with the LA and the crista terminalis with the pectinate muscles by multiple pacing protocols. Re-entry can be initiated at both these heterogeneous junctions in the atria, with the PV/LA junction exhibiting the largest vulnerability; rapid pacing, S1S2 stimuli and short-lived re-entry in the right atrium all lead to the development of re-entrant activity in the PV/LA region. This high vulnerability is a result of a combination of short action potential durations and significant heterogeneity, which are both accentuated in the remodelled atria. This study provides mechanistic insights to the understanding of the high vulnerability of tissue to formation of re-entry in the PVs.

III.P2.

Contractile dysfunction of left ventricular cardiomyocytes in patients with pulmonary arterial hypertension

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Rational A reduction in left ventricular ejection fraction and free wall mass in patients with pulmonary arterial hypertension (PAH) has been reported. However, it is unknown whether changes in cardiomyocyte function and structure underlie the reduced LV function and mass.
Methods Cardiomyocyte cross sectional area (CSA) was determined in cryosections (anti-laminin) of LV free wall of end-stage PAH-patients

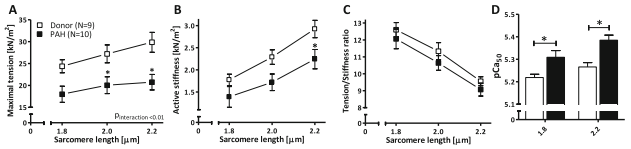

Fig. 1 **a** Maximal tension, **b** number of cross bridges, **c** force per cross bridge and **d** calcium sensitivity

(n = 10) and donor hearts (n = 9). Subsequently, triton-permeabilized cardiomyocytes were mounted between a force transducer and length motor. Maximal active tension (F_{max}, normalized to CSA) was measured at saturating Ca^{2+}-concentration (pCa4.5), while Ca^{2+}-sensitivity (pCa_{50}) was determined at incremental Ca^{2+}-concentrations.
Active stiffness was measured by imposing small length steps on the cardiomyocyte while in activating solution (pCa4.5).
Results Cardiomyocyte CSA (normalized to a sarcomere length (SL) of 2.0 µm) was significantly smaller in PAH-patients (Donor: 406 ± 48, PAH: 279 ± 21, $p < 0.05$). F_{max} was significantly lower in LV cardiomyocytes from PAH-patients (Fig. 1a), while passive tension was not different between groups. Active stiffness, reflecting the number of cycling cross-bridges, was significantly reduced at SL of 2.2 µm in PAH-patients (Fig. 1b). The tension/stiffness ratio, reflecting the force per cross-bridge, was not different between groups (Fig. 1c). pCa_{50} was significantly increased at both 1.8 and 2.2 µm SL (Fig. 1d).
Discussion We demonstrated that LV cardiomyocytes of PAH-patients are atrophic, generate less force at maximal activation, but have increased Ca^{2+}-sensitivity. The observed changes in LV cardiomyocyte function and structure might underlie the post-operative complications observed in PAH-patients after a lung-transplant, when the LV is suddenly re-exposed to high filling pressures.

III.P3.

Mena/VASP-dependent anchoring of cytoplasmic actin protects against dilated cardiomyopathy

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Regulation of *cytoplasmic* actin dynamics is vitally important for the mechanical stability and contractility of heart muscle cells. The proteins Mena and VASP are important regulators of actin dynamics. Due to the lethal phenotype of Mena/VASP double-deficient mice, redundant and essential cardiac functions of the proteins are unclear and molecular mechanisms remain speculative. The aim of this study was to investigate if Mena and VASP have unique or overlapping functions in the mammalian heart and how these proteins contribute to cardiac performance. Here, we generated a unique mouse model, which simultaneously lacks both proteins in the heart. Physiological analyses demonstrated an increasingly impaired left ventricular performance in VASP-, Mena-, and Mena/VASP double-deficient animals, which developed dilated cardiomyopathy (DCM) and ventricular conduction abnormalities.
We found that Mena and VASP specifically interact with a distinct α II Spectrin splice variant (SH3*i*), which is exclusively localized at Z- and intercalated discs of cardiomyocytes. Detailed immuno-electron microscopical analysis of the dKO and WT cardiac cytoarchitecture revealed a phenotype with structural alterations of the sarcomers and the intercalated discs.
Our data suggest that Mena, VASP, and SH3*i* assemble cardiac multiprotein complexes, which promote *cytoplasmic* actin dynamics to ensure force transduction and propagation of electrical signals. Conversely, Mena/VASP double deficiency impairs left-ventricular performance in vivo and results in dilated cardiomyopathy and ventricular conduction abnormalities.

III.P4.

Changes in myofibrillar Ca^{2+}-sensitivity with low-frequency force depression

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Low-frequency force depression (LFFD) is characterized by reduced tetanic force at low frequencies of stimulation while tetanic force at high stimulus frequencies is close to normal. The purpose of this study was to examine changes in myofibrillar Ca^{2+}-sensitivity in fast-twitch muscle fibers with LFFD. Intact rat gastrocnemius muscles (GS) were electrically stimulated (70 Hz, 0.35-s train, every 3 s) via the sciatic nerve until force was reduced to 50 % of initial. Tetanic force in intact GS was measured 30 min after the end of fatiguing stimulation. Mechanically skinned fibers were then prepared from the superficial region of GS. The ratio of tetanic force at 20 Hz to that at 100 Hz fell to 75 % after 30 min. Skinned fibers were directly activated in heavily buffered Ca^{2+}-solutions to assess myofibrillar Ca^{2+}-sensitivity. Maximum force or pCa_{50}, a concentration at half-maximum force, were unaltered in fatigued muscle fibers. When skinned fibers were subjected to *S*-glutathionylation by successive treatments with 2,2′-dithiodipyridine and glutathione, pCa_{50} was more increased in unfatigued fibers than in fatigued fibers. These findings suggest that decreases in myofibrillar Ca^{2+}-sensitivity are not responsible for LFFD at least in an in situ fatigue model and that *S*-glutathionylation that occurs during muscle contractions would offset the depressive effect of contraction to fatigue on the Ca^{2+}-sensitivity.

III.P5.

Sphingolipid profile in the unifarcted ventricular wall of the rat after myocardial infarct

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Myocardial infarction is accompanied by increased apoptosis of cardiomyocytes in the uninfarcted area of the left ventricle of the rat. It is well established that the ratio between the level of ceramide and the level of sphingosine-1-phosphate determines cardiomyocytes survival. The aim of the present study was to investigate the effect of myocardial infarction on the level of different sphingolipids in the uninfarcted area of the left ventricle. The experiment was carried out on the male Wistar rats (200–220 g of body weight). Animals were divided into three groups: 1—Control group, 2—Infarct group (after ligation of the left coronary artery) and 3—Sham operated group. Samples of the uninfarcted area of the left ventricle were taken in 1, 6, and 24 h after the surgical procedure. The level of sphingosine-1-phosphate, ceramide, sphingosine, sphinganine-1-phosphate and sphinganine was measured by means of an Agilent 6460 triple quadrupole mass spectrometer.
In Infarct group the level of sphingosine-1-phosphate in the uninfarcted area was reduced by ~3 times in 1 and 6 h and decreased further in 24 h. The level of sphingosine decreased in 1 h and thereafter returned to the Control level. The level of sphinganine remained unchanged whereas the level of sphinganine-1-phosphate decreased in 1 h and further remained stable. The total level of ceramide decreased in 24 h after the infarction. The ratio between the level of sphingosine-1-phosphate and the level of ceramide was markedly reduced at each time point after the myocardial infarction. It

can be concluded that the reduction in the sphingosine-1-phosphate/ceramide ratio could be responsible for increased apoptosis observed in the uninfarcted area of the left ventricle after the myocardial infarction in the rat.

POSTER SESSION IV Excitation–contraction coupling

IV.P1.

Temperature and 'fatigue' effects on ATP-induced dissociation of fast and slow actomyosin

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Fast and slow mammalian-muscle myosins differ in their heavy chain sequences (MHC-2 MHC-1) and muscles expressing the two isoforms contract at markedly different velocities. Nucleotide affinity is also affected by MHC isoform, with slow isoforms showing tighter ADP binding to A.M and slower ADP release rates than fast isoforms. In vivo muscle temperature ranges from 34 to >40 °C and in extreme fatigue the pH may drop substantially and inorganic phosphate (Pi) accumulates. Recent fibre mechanics data indicate that these effects of fatigue and temperature may differ in the different fibre types. We examined the effects of pH (7 vs. 6.2), and Pi (0–30 mM), singly or in combination on the kinetics of ATP-induced dissociation of A.M. (±ADP) of rabbit fast and slow myosin over a range of temperatures (5–35 °C). Neither temperature nor pH had a strong effect on ADP affinity for fast myosin, but weakened affinity in slow myosin at low pH and high temperature. Phosphate competes with ADP binding to fast A.M, but has little effect on ADP binding in slow A.M. Displacement of ADP from slow A.M is biphasic. Temperature dependence of the two phases of ADP release from A.M in slow myosins is similar, with little effect of phosphate or pH (10 %). While the pH had little effect on the two phases at differing temperatures, phosphate caused a marked loss of fast phase amplitude at high temperature, suggesting that Pi competes with ADP for slow A.M at high temperatures.

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IV.P2.

Studies of actin conformational states related to myosin binding

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It has been shown that, the statistics of the winding path of actin filaments propelled by heavy meromyosin (HMM) in the in vitro motility assay (IVMA) can be used to estimate the flexural rigidity of the free leading end of the filament. The persistence length, L_P, of a polymer describes the decay length of the polymer tangent angle and is proportional to the flexural rigidity and can thus be used as a measure of this material property of the actin filament. We measured L_P of actin filaments under different conditions in order to address a hypothesis that the actin filament exists in different metastable conformations each characterized by a different flexural rigidity. The measurements were performed using HMM propelled actin filaments in the IVMA at different MgATP concentrations and filaments free in solution in the presence and absence of HMM and subfragment 1 (S1). Our results show a decreased L_P of actin filaments at low MgATP concentrations in the IVMA and also a HMM concentration dependent decrease of L_P in filaments free in solution. Furthermore, we will also describe Monte-Carlo simulations of winding filament paths of HMM propelled actin filaments to elucidate the impact of experimental complications, e.g. sideways filament motion, induced by HMM attachment on the actin filament sliding direction.

IV.P3.

Similarities in the regulation of insect flight muscle and cardiac muscle of vertebrates

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The indirect flight muscle (IFM) of insects and cardiac muscle contract rhythmically. Both have marked length-dependent activation. In cardiac muscle, force developed at each beat depends on extension of the fibres, and this is enhanced by brief stretch-activation. In IFM, periodic stretch-activation produces high frequency oscillatory contractions. In both IFM and cardiac muscle, thin filaments are involved in the response to changes in muscle length. TnC in cardiac muscle binds one regulatory Ca^{2+} in the N-lobe. The isoform of TnC (F1) in IFM needed for stretch-activation binds one Ca^{2+} in the C-lobe; a minor isoform (F2) binds an additional Ca^{2+} in the N-lobe and is responsible for isometric force. Force-pCa curves for IFM with F2 only are similar to those of cardiac muscle, with $pCa_{50} = 5.8$ and cooperativity, $n_H = 3.2$. Force development by fibres with F1 and a small proportion of F2, is more sensitive to Ca^{2+} ($pCa_{50} = 6.2$ and less cooperative ($n_H = 1.3$). Thus, oscillatory contractions occur at Ca^{2+}-concentrations where there is little isometric force. The N-lobe of F1 is necessary for stretch-activation. The C-lobe (F1-Ct) inhibits stretch-activation by displacing F1 from the thin filament; F1-Ct can be displaced by F2, restoring isomeric force, but not stretch-activation. In *Drosophila*, F1 and F2 can be independently down-regulated by RNAi. Flies with F1 fly normally but those with F2 are flightless. In fibres without F2, Ca^{2+}-sensitivity of work is reduced and less cooperative. It is likely there are parallels in TnC function in stretch-activation of IFM and cardiac muscle.

IV.P4.

Dynamic coupling of regulated binding sites and cycling cross-bridges produces activation-dependent mechanical properties in muscle

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In an activated muscle, binding sites on the thin filament and myosin heads switch frequently between different states. Since the status of the binding sites influences the status of the heads, and vice versa, the thick and thin filaments are dynamically coupled. The functional

significance of this coupling depends on the relative speeds of the kinetic processes and it can therefore be challenging to predict contractile properties from data and/or assumptions about the behavior of individual proteins. A new model of muscle, called MyoSim, has been developed to accelerate progress in this area. MyoSim is an implementation of a Huxley-type distribution model that has been extended to incorporate Ca^{2+}-activation, cooperative effects, and a variety of cross-bridge schemes. The source code, along with tutorials and examples, can be downloaded (GPL license) at http://www.myosim.org. Initial calculations used parameter optimization to fit simulated force records to experimental data recorded using permeabilized myocardial preparations. These simulations suggest that the rate of force development at maximum activation is limited by myosin cycling kinetics while the rate at lower levels of activation is limited by how quickly the binding sites become available. Additional simulations showed that, when driven by an experimentally recorded Ca^{2+}-transient, a MyoSim model with two cross-bridge states and strain-dependent kinetics can reproduce the unloaded shortening profile of a single cardiac myocyte.

IV.P5.

Low temperature and transmural effects augment variability in measurements of calcium transients and sarcomere length in unloaded left ventricular cardiomyocytes

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Calcium transients and sarcomere length contraction/relaxation profiles can be measured from intact cardiomyocytes to assess cellular level contractility and, potentially, to help screen new therapies. Reducing the variance of the experimental data would improve the efficiency of the technique. We hypothesized (1) that temperature affects variance in these measures and (2) that cells from different regions would have different properties, which could also add to variance. Intact cardiomyocytes were isolated from the subendocardium, midmyocardium and subepicardium of 3 month old Sprague-Dawley rats. Myocytes from each region were studied at 25, 31 and 37 °C. For both the calcium transients and sarcomere length profiles, the time to peak and the time to the 50 % decay decreased significantly with increasing temperature. Importantly, the variance of these measurements also decreased. Increasing temperature raised the minimum and maximum calcium levels of all cells, but the magnitude and minimum of the calcium transient were also significantly dependent on transmural region. This suggests that performing experiments with cells that are randomly selected from different parts of the ventricle will increase the variance of the data because it ignores significant transmural effects. Additionally, there were significant statistical interactions between temperature and transmural region for maximum sarcomere length shortening. This implies that temperature affects cells from the three transmural regions in different ways. In summary, our data suggest that experiments testing potential therapies should be performed at 37 °C to minimize variance. Additionally, our data suggests that using cells from one transmural region will reduce variance and increase statistical power.

IV.P6.

The effects of steady lengthening on force of rat soleus fibres: data to test models of cross-bridge function

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Although there are existing models of cross-bridge kinetics during stretching there are some features of the force responses to stretch which may be used not only to develop and test future models but also suggest that existing models may need to include the involvement of the second myosin head during stretching[3].

The procedures for the determination of single fibre contractile properties at 15 °C from soleus muscles of adult male Wistar rats have been described previously.

Return to pre-stretch force at the end of a steady stretch was best fitted by a double exponential with the rate constant for the slow phase being between 6 and 12 s^{-1} and showing little variation with speed of the stretch. The fast component, however, increased from 23 ± 3 to 45 ± 3 and 121 ± 2 s^{-1} after stretches at 0.0625, 0.25 and 1*lo* s^{-1} while the proportion of force accounted for by the fast component also increased from 32 to 45 and 54 %. Following a 0.5 % *lo* step increase in length superimposed on the steady stretching, force returned to the value sustained during the steady stretch with a time course best fitted by a single exponential, with rate constants of 65 ± 12, 311 ± 51 and 543 (442,645) s^{-1} during slow, medium and fast stretches, compared with 13 ± 1 s^{-1} for an isometric contraction.

These observations are consistent with cross-bridge intermediate states with very high rates of turnover during stretching. It remains to be seen how well new models of cross-bridge kinetics can explain these features.

IV.P7.

Effect of mechanical factors on the rhythm disturbances in cardiomyocytes overloaded with calcium: modeling and experimental validation

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It is well-known that Ca^{2+}-overload may cause cardiac arrhythmia. However, possible contribution of the mechanical factors to the arrhythmia onset in Ca^{2+}-overloaded cardiomyocytes has been poorly investigated. Our mathematical model predicts that mechanical factors do underlie this onset. The modeling showed that dependency of calcium-troponin kinetics on force generating cross-bridge concentration (cooperativity mechanism) played a role of trigger for extrasystoles and other rhythm disturbances associated with the overload of cardiomyocytes with calcium. Besides, according to the model prediction the following mechanical factors turned out to promote arrhythmogenesis in such cardiomyocytes: (i) a decrease in the mechanical load for afterloaded contractions; (ii) a decrease in the initial length of sarcomeres for isometric twitches, (iii) mechanical interactions between myocardial segments (clusters of cardiomyocytes) with different levels of Ca^{2+}-loading. The model predictions were verified in experiments on papillary muscles from the right ventricle of guinea pigs with Ca^{2+}-overloaded cardiomyocytes (ouabain (0.5–1 μM) was used to obtain the Ca^{2+}-overload mediated by reduction in Na^{+}–K^{+} pump activity). The experiments confirmed emergence of rhythm disturbances depending on mechanical conditions of the heart muscle contractions in conformity with the modeling.

IV.P8.

Force produced by thick filaments isolated from soleus muscles lacking arginyl-tRNA-protein transferase (Ate1)

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Introduction The enzyme arginyl-tRNA-protein transferase (Ate1) is responsible for transferring arginine (Arg) from tRNA onto proteins. Data from our laboratory suggests that arginylation is a major regulator of skeletal muscles. In this study, we evaluated if lack of arginylation affects the velocity of myosin-propelled actin motility, and the force produced by skeletal muscle thick filaments while interacting with actin filaments.
Methods Soleus muscles from Ate1 KO and wild type (WT) mice were used to obtain the isolated thick filaments. Actin filaments were polymerized from cardiac actin proteins. Micro-fabricated cantilevers were used to measure the force produced during interactions between thick and actin filaments. In-vitro motility essays were used to measure the myosin-propelled velocity of actin filaments. Myosin heavy chain (MHC) analyses were performed to identify potential shifts in isoforms.
Results Thick filaments isolated from Ate1 KO mice developed less force when compared to filaments isolated from WT mice (111.37 ± 8.01 pN/0.8 μm and 137.32 ± 7.45 pN/0.8 μm, respectively). When normalized per number of myosin cross-bridges in the filaments, the forces were 3.98 ± 0.28 for the Ate1 KO and 4.91 ± 0.26 for the WT mice, respectively. No difference was found in the myosin-propelled actin velocity (Ate 1 KO 1.14 μm/s $\pm$ 0.03 and WT $= 1.09 \pm 0.04$ μm/s).
Conclusion Thick filaments from Ate1 KO mice produce less force than the filaments from WT mice, suggesting that arginylation regulates force production in skeletal muscles by affecting the unitary force produced during myosin-actin interactions.

IV.P9.

The cytoplasmic foot of type 1 ryanodine receptor targets junctionally, retrogradely enhances l-type Ca^{2+}-currents and homotetramerizes

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The skeletal muscle type 1 ryanodine receptor (RyR1; 5,037 residues) forms a homo-tetrameric Ca^{2+}-release channel in the sarcoplasmic reticulum (SR), mediates excitation–contraction coupling in response to an orthograde signal from the dihydropyridine receptor (DHPR) in the plasma membrane, and retrogradely enhances L-type Ca^{2+}-current via the DHPR. The RyR1 C-terminus contains the Ca^{2+}-channel pore across the SR membrane and is believed to be important for inter-subunit interactions, whereas the bulk (~ 85 %) of the protein (the so-called foot) bridges the junctional, myoplasmic gap between the SR and plasma membranes. Here, we examined the ability of the foot domain (residues 1–4,300; $RyR1_{1:4,300}$) to target junctionally by expression of YFP-$RyR1_{1:4,300}$. In dysgenic (α_{1S}-null) myotubes which lack DHPRs, YFP-$RyR1_{1:4,300}$ was diffusely distributed and, on the basis of FRAP, freely mobile within the cytoplasm with a diffusion coefficient of 2.17×10^{-8}cm^2/s, compatible with YFP-$RyR1_{1:4,300}$ existing as a tetramer. However, after expression in dyspedic (RyR1 null) myotubes (which have DHPRs), YFP-$RyR1_{1:4,300}$ was often immobile within fluorescent foci near the myotube surface and partially co-localized with the DHPR. Strikingly, YFP-$RyR1_{1:4,300}$ was able to retrogradely enhance peak Ca^{2+}-current in dyspedic myotubes from 1.6 pA/pF (control) to 6.7 pA/pF, similar to that after expression of full-length RyR1 (7.4pA/pF). Immunoblotting in combination with chemical cross-linking revealed that YFP-$RyR1_{1:4,300}$, much like wt full-length RyR1, in 4–15 % SDS gradient gels migrates as single band of high apparent MW, consistent with it's being a tetramer.

IV.P10.

Temperature dependence of myosin filament structure in relaxed mammalian muscle

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X-ray patterns from relaxed skinned fibres from rabbit psoas were compared with those from intact EDL muscle of mouse at rest. At 10 °C, the M3 reflection from the axial repeat of the myosin heads indicated a spacing $S_{M3} = 14.45 \pm 0.02$ nm in the skinned fibres and $S_{M3} = 14.34 \pm 0.02$ nm in the EDL, and showed a fine structure with the lower angle peak being a fraction of the total intensity $L_{M3} = 0.63 \pm 0.04$ and $L_{M3} = 0.33 \pm 0.04$, respectively. In both preparations, increasing temperature reduced the intensity of the lower angle peak, and at 35 °C M3 showed a main peak at 14.35 nm, with small satellite peaks on either side. It also increased the intensity of the forbidden M2, M4, M5 and of the M6 meridional reflections. Addition of an osmotic agent in the skinned fibres to recover the lattice spacing of the intact muscle increased the intensity of the meridional reflections and induced a change in the fine structure of the M3 similar to that observed increasing temperature. Increasing the sarcomere length from 2.4 to 3.4 μm in skinned fibres at 25 °C reduced the intensity of the forbidden reflections and increased the spacing of the M2, M3 and M6 reflections. The effects of sarcomere length and osmotic compression on the temperature-induced structural changes in relaxed skinned fibres constrain structural models for the OFF state of the myosin filament and for the changes in myosin filament structure on muscle activation.

IV.P11.

New evidence for calcium mishandling in human Calpain 3 deficient myotubes

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Limb girdle muscular dystrophy type 2A (LGMD2A) is one of the most frequently occurring forms of recessive muscular dystrophies and it is characterized by primary wasting of scapular and pelvic muscles that result in progressive muscle weakness. Impairment of calcium transport is one contributing factor to the clinical phenotype of LGMD2A muscular dystrophy, which is caused by mutations in Calpain 3 (CAPN3), a non-lysosomal calcium-dependent cystein protease. As we have recently shown in a collaboration study involving CAPN3 knockout mice and LGMD2A patients, impaired calcium-mediated signaling and weakened muscle adaptation are pathogenic mechanisms operating in CAPN3-deficient muscles. In the present study, we have analyzed using real time PCR, western

blotting and calcium imaging techniques a whole array of molecules implicated in calcium handling in skeletal muscle fibers and found significant reduction in a number of proteins essential for calcium homeostasis. Our new findings in human myotubes are in line with the ones previously described in the LGMD2A mouse model and suggest novel targets that open new avenues for potential therapeutic approaches to treat LGMD2A muscular dystrophy.

Monday September 23, 2013

POSTER SESSION V Large cytoskeletal proteins

V.P1.

Determining the molecular mechanisms that link a titin mutation to cardiomyopathy

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A mutation in the giant elastic protein titin has recently been linked to arrhythmogenic cardiomyopathy (AC), a disease primarily characterized by fibrofatty infiltration of the myocardium. Titin is the first sarcomeric protein linked to AC. In addition to largely unstructured segments, the extensible I-band region of titin contains ~40 immunoglobulin (Ig)-like domains that are natively folded as highly stable beta barrels. One of these domains, Ig10, contains a single Thr → Ile mutation that leads to AC. The first step in determining how this mutation leads to severe cardiac disease was to study the mutation at the single molecule level using atomic force microscopy (AFM) and in vitro degradation assays. With AFM individual proteins are mechanically stretched, and it was found that the mutation significantly reduces the force needed to unfold Ig10. Although no difference in refolding rate was found, by comparing the experimental AFM unfolding forces with computer simulated Ig unfolding, it was found that the mutation increases Ig10's unfolding rate fourfold. This effect predicts increased Ig10 unfolding (i.e. transition into a non-native state) under physiological conditions. It was also found that mutant Ig10 is more prone to protease degradation due to compromised local structure at the point mutation. This suggests that accelerated titin turnover in cardiomyocytes instigates the cardiac remodeling process in patients with titin-linked AC. This is the first time an Ig domain in titin's elastic I-band region has been linked to cardiac disease, and this study proposes a novel disease mechanism contingent on Ig domain unfolding.

V.P2.

Study on amyloidogenesis of smooth muscle and striated muscle titin in vitro

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Amyloid deposits, unlimitedly growing in various organs and tissues, play a central role in the pathogenesis of the large numbers diseases (Alzheimer's disease, Parkinson's disease, type 2 diabetes, systemic amyloidoses, etc.). The processes underlying the anomalous protein aggregation to amyloids and their pathological manifestation in disease are still insufficiently studied. Amyloid fibrils have some common properties: β-structure with separate β-sheets which are parallel to the fibril main axis; in vivo insolubility; specific staining with Congo Red and thioflavin T.

Using electron microscopy we showed that rabbit T2-fragment of titin formed bundles of fibrils (~3 μM long, 500 nm in width), and amorphous aggregates in solution containing 0.15 M glycine–KOH, pH 7.5. Smooth muscle titin and its 500 kDa fragment formed amorphous aggregates and bundles of fibrils in the solutions containing 0.15 M glycine–KOH, pH 7.0, and 0.05 M glycine–KOH, pH 7.0. Amyloid nature of the aggregates of titin from striated and smooth muscle was confirmed by spectroscopic methods using dyes for amyloid (Congo red and thioflavin T). According to the preliminary data 500 kDa fragment of the smooth muscle titin has a toxic effect on the culture of smooth muscle cells, which may indicate to their possible pathological roles in vivo.

V.P3.

Removal of titin's proximal Ig domains results in differential splicing of titin and alters skeletal muscle function

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Titin is the largest protein known and functions as a molecular spring in striated muscle. Its elastic properties are acquired through differential splicing of spring-like domains of titin including the immunoglobulin-like (IG) and PEVK. Our lab has created a novel mouse model in which nine of the proximal IG domains (titin exons 30–38, 90 kDa reduction in protein size) have been deleted (IG KO). Surprisingly in the skeletal muscles from IG KO mice, titin was found to undergo additional differential splicing to yield smaller titin isoforms. Specifically, in the adult soleus muscle, two smaller titin isoforms were present in the IG KO (3.42 and 3.24 MDa, respectively) as compared to one larger isoform found in wild-type mice (3.61 MDa). The titin splicing factor RBM20 was significantly upregulated in soleus muscle from IG KO mice (2.1-fold). Consistent with a decrease in titin size, IG KO muscles produced a significant increase in passive tension as probed by both intact and skinned mechanics. Soleus muscle had a significant decrease in twitch force (33.6 vs. 19.0 mN/mm^2) was more resistant to fatigue, and had an increase in the expression of myosin I (2.7-fold increase). The additional alternative splicing was found to be developmentally regulated and correlates with changes in the size and expression of the titin binding protein CARP. This model provides an exciting opportunity to explore the mechanisms that underlie titin alternative splicing and how alternative splicing alters muscle function.

V.P4.

Shortening of the proximal tandem Ig segment of titin leads to diastolic dysfunction

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Diastolic dysfunction is a poorly understood but clinically pervasive syndrome that is characterized by increased diastolic stiffness. Titin is the main determinant of cellular passive stiffness. However, the physiological role that the tandem immunoglobulin (Ig) segment of titin plays in stiffness generation and whether shortening this segment is sufficient to cause diastolic dysfunction need to be established. Methods and Results: We generated a mouse model in which 9 Ig-like domains (Ig3–Ig11) were deleted from the proximal tandem Ig segment of the spring region of titin (IG KO). Exon microarray analysis revealed no adaptations in titin splicing, whereas novel phospho-specific antibodies did not detect changes in titin phosphorylation. Passive myocyte stiffness was increased in the IG KO, and immunoelectron microscopy revealed increased extension of the remaining titin spring segments as the sole likely underlying mechanism. Diastolic stiffness was increased at the tissue and organ levels, with no consistent changes in extracellular matrix composition or extracellular matrix–based passive stiffness, supporting a titin-based mechanism for in vivo diastolic dysfunction. An age-dependent hypertrophy is observed and likely related to an increase in FHL1 signaling. Additionally, IG KO mice have a reduced exercise tolerance, a phenotype often associated with diastolic dysfunction. In conclusion, increased titin-based passive stiffness is sufficient to cause diastolic dysfunction with exercise intolerance.

V.P5.

Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat cardiometabolic risk model

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Background Obesity, diabetes and arterial hypertension are key constituents of cardiometabolic risk (CMR) and frequent comorbidities in HFPEF. They can induce myocardial diastolic dysfunction (DD) through collagen deposition or titin modification. The relative importance of these two mechanisms for HFPEF associated with CMR remains uncertain.
Methods Obese Zucker spontaneously hypertensive diabetic fatty rats were fed with normal (ZSF1 Obese, n = 11) or high fat diet (ZSF1 Obese + HFD, n = 11). Hypertensive lean ZSF1 (ZSF1-Lean, n = 11) and Wistar Kyoto (WKY, n = 11) rats served as controls. Obese rats had developed HFPEF, evident from echocardiographic, invasive and morphometric assessment at 20 weeks of age. Resting tension (Fpassive)-sarcomere length relations were obtained in isolated cardiomyocytes (LV free wall) and in muscle strips (LV papillary muscle) before and after KCl-KI treatment, which unanchors titin and allows contributions of titin and extracellular-matrix (E-matrix) to Fpassive to be discerned. Expression and phosphorylation of titin were assessed by electrophoresis.
Result In strips, total Fpassive was increased in both obese groups. E-matrix based Fpassive was similar in all groups at sarcomere lengths 1.9–2.2 μm, but rose sharply at higher lengths. Titin based Fpassive was increased in obese rats. Fpassive was higher in single cardiomyocytes from obese rats compared to controls and in vitro protein kinase G (PKG) administration lowered Fpassive to control values. No titin isoform-shifts were observed, but titin was hyphosphorylated in obese rats.
Conclusion This CMR-rat model is associated with HFPEF at 20 weeks of age. The underlying DD mainly results from a titin phosphorylation deficit, which is more prominent than changes in the E-matrix.

V.P6.

Human myocytes are protected from stress-induced stiffening by chaperone binding to titin spring elements

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Small heat-shock proteins (sHSPs) are abundant in myocytes where, under stress conditions, they translocate preferentially to the sarcomeres. The functional implications of this translocation are incompletely understood. We hypothesized that sHSPs bind to human titin-spring elements and exert a protective effect on myocyte passive stiffness under stress conditions. We characterized binding of HSP27 and αB-crystallin to recombinant human titin constructs by GST-pulldown assay and binding to sarcomeres by indirect immunofluorescence. Sedimentation-velocity centrifugation, photometric, and chromatographic methods were used to measure titin aggregation. Passive sarcomere length-tension relationships were recorded in isolated permeabilized human cardiomyocytes while buffer-pH was varied, in the presence or absence of sHSPs. We found that HSP27 and αB-crystallin share binding sites on the titin springs, associating with immunoglobulin-like (Ig-) domain-containing regions (proximal-Ig/N2-B/N2-A), but not with the intrinsically disordered PEVK-domain. In sarcomeres, sHSP-binding to the titin springs occurred independent of the presence of actin filaments and was enhanced by stretch. The titin-spring elements behaved in vitro predominantly as monomers. However, unfolded Ig-regions aggregated preferentially under acidic conditions, whereas αB-crystallin protected against aggregation. N2-B and PEVK did not aggregate. In single human cardiomyocytes pre-stretched under acidic stress, titin-based stiffness was overly high but HSP27 or αB-crystallin prevented this stiffening. In muscle/myocardial tissue from patients with muscular dystrophy (calpainopathy) or heart failure (dilated cardiomyopathy), both sHSPs associated with the elastic I-band, in contrast to their cytosolic or Z-disk location in healthy human muscle/heart. We conclude that sHSPs translocate to the titin springs to prevent stress-induced titin aggregation and abnormal myocyte stiffening.

V.P7.

Filamin C is a highly dynamic Z-disc protein immobilized by Xin and Xirp2

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Z-discs define the lateral borders of the sarcomere in striated muscles and have primarily been considered to be important for mechanical stability. A multitude of novel Z-disc proteins including filamin C suggest additional functions such as intracellular signaling, mechanosensation and mechanotransduction. Mutations in Z-disc proteins may lead to severe muscle diseases.
FLNc is comprised of an actin-binding domain followed by 24 immunoglobulin-like domains the last of which is responsible for dimerization, while domain 20 contains a unique insertion of 80 aa.

Previously we reported that FLNc domains 20–21 serve as scaffold for many binding partners such as Xin, myotilin and myopodin. We now discovered Xin actin-binding repeat containing protein 2 (Xirp2) to be a novel FLNc ligand that is able to bind FLNc simultaneously with Xin. To investigate its mobility in living cells we expressed FLNc as EGFP-fusion protein in mouse embryonic cardiomyocytes and applied fluorescence recovery after photobleaching (FRAP). The half-life ($t_{1/2}$) of FLNc in mature Z-discs is biphasic and surprisingly short. 80 % of FLNc is in the mobile fraction, indicating a high turnover rate. In non-striated pre-myofibrils the $t_{1/2}$ is significantly lower, while only 50 % of the protein is mobile. A FLNc variant lacking the insertion in domain 20 showed significantly increased dynamics in the Xin and Xirp2-containing pre-myofibrils, but not in mature Z-discs where both ligands are absent. Furthermore, in cardiomyocytes derived from mice deficient for Xin and Xirp2, FLNc dynamics in the pre-myofibrils were significantly increased from that in wild type cardiomyocytes, with half-life and mobility being as high as in the Z-discs of wild-type cells. These results indicate that the interaction of Xin and Xirp2 with domain 20 of FLNc plays an important role in stabilizing FLNc in pre-myofibrils.

V.P8

Evidence that H-zone width in vertebrate muscle sarcomere is quantised in steps of 43 nm

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Sections of relaxed vertebrate muscle A-bands show eleven distinct stripes of spacing 43 nm starting from the bare zone. Stripes 5–11 in fast skeletal muscle and 3–11 in cardiac and slow skeletal muscle are due to myosin binding protein C (MyBP-C). We have shown that the elongated MyBP-C molecule in fast skeletal muscle makes a perpendicular connection from the thick filaments to the thin filaments, in agreement with the narrow stripe widths. We propose that the proteins on stripes 1–4 also bind to actin. We would expect that changing the sarcomere length might lead to wider, less dense stripes. However, we observe strong stripes at different sarcomere lengths. We conclude that MyBP-C detaches from actin and reattaches at the new stripe position. If the position of MyBP-C is not properly matched to actin, then it is possible that MyBP-C influences actin to move to the optimal binding position. Using electron micrographs of well-preserved frog fast muscle, we have investigated the relationship between the edge of the H-zone (defined by the ends of the actin filaments) and the positions of the stripes at three different sarcomere lengths. Averaged density profile plots of half A-bands for each sarcomere length often show sloping H-zone boundaries between two stripes but more frequently show a distinct step at a stripe location. Hence the H-zone width often varies in steps of 43 nm, suggesting that MyBP-C influences thin filament position. This gives additional evidence that MyBP-C binds to thin filaments.

V.P9.

Large-scale molten-globule dynamics contribute to titin contractility

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When exposed to high forces, titin unfolds in discrete steps via unfolding of its globular domains. By contrast, refolding is strongly inhibited by mechanical force and chain extension, which results in a large force hysteresis in stretch-relaxation cycles. Whereas the stretch force curve of titin is populated by sawtooth-shaped transitions, the relaxation force curve is devoid of significant transitions, making it difficult to capture the refolding event. Although titin refolds if incubated in the contracted state, the exact trajectory of the folding process is unclear. To explore the detail of titin's mechanically-driven folding and unfolding, we manipulated single molecules with high-resolution optical tweezers. Whereas titin extended in discrete steps at high constant forces, after quenching the force to low levels the extension fluctuated without resolvable discrete events but with low frequency (second timescale) and high (several 100 nm) peak-to-peak amplitude. In constant-trap-position experiments at very low (<1 pN) average forces fluctuations were observed, suggesting that the domains hop between an extensible unfolded and a compact molten-globule state. Monte-Carlo simulations based on a compact molten-globule intermediate recovered all features of the force-clamp results. Under mild denaturing conditions (0.5 M urea) that favor the molten-globule state, the length and force fluctuations appeared even in constant-velocity experiments, indicating that this intermediate is part of the folding trajectory. Because the transition from the unfolded to the molten-globule state shortens the chain faster than a purely entropic collapse, an additional sarcomeric contractility may arise under stressed conditions when titin's domains become unfolded.

V.P10.

Low-force structural transitions in single titin molecules reflect a memory of contractile history

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Mechanical forces are increasingly being recognized to influence a wide array of cellular and biomolecular processes. These mechanobiological phenomena require the sensing of the local mechanical environment, the mechanisms of which are vaguely and poorly understood. It is hypothesized that in striated muscle the giant elastomeric protein plays the most important role in the mechanosensory process. To explore how titin responds to physiologically relevant forces on different temporal and spatial scales, we manipulated single molecules, purified from rabbit skeletal and cardiac muscles, by exposing them to complex nanomechanical scenarios with high-resolution velocity- or force-clamp optical tweezers. Unexpectedly, titin extended via discrete transitions even at forces as low as 5 pN, which increased the molecular contour length by steps ranging between ten to several hundred nanometers. Force-clamp experiments indicated that the rate constant of the low-force transitions far exceeds that of I27-domain unfolding. Ionic-strength-dependent, 9D10-antibody-passivation and isoform-comparison (skeletal vs. cardiac) experiments suggested that the site of the transitions may be allocated to the PEVK domain and to a small number of mechanically weak globular domains in the proximal tandem-Ig region of titin. Although the intrachain interactions behind the low-force transitions are highly dynamic and are even able to contract titin against mechanical force, the recovery and global pattern of the transitions are strongly influenced by the time- and extension-dependent mechanical history of the molecule. We thus hypothesize that titin senses the mechanical environment by a self-zippering process the kinetics of which are tuned by sarcomeric extension and force exposure.

V.P11.

Actin and tropomyosins show different binding affinities to mutant nebulin in vitro

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Nemaline myopathy (NM) is a rare genetic muscle disorder defined on the basis of muscle weakness and the presence of structural abnormalities in the muscle fibres, i.e. nemaline bodies. NM is caused by mutations in at least seven genes: Nebulin (NEB), Actin (ACTA1), α-tropomyosin (TPM3), β-tropomyosin (TPM2), troponin T (TNNT1), cofilin 2 (CFL2) and KBTBD13. Nebulin (NEB) is a giant 600–900 kDa filamentous protein that is a part of the skeletal muscle thin filament. It has been shown that ~90 % of the primary structure of NEB is composed of ~35-residue α-helical simple repeat domains that bind actin. Most of the simple repeats are arranged into seven-module super repeats. Each super repeat contains a putative tropomyosin-binding site.

In order to study the effects of mutations in tropomyosin and actin on the bidning of these proteins to NEB, we have produced four wild-type (wt) nebulin super repeats (283–347 amino acid long) and five corresponding mutants. Three missense mutations (p.Glu2431Lys, p.Ser6366Ile and p.Thr7382Pro) and two in-frame deletions (p.Arg2478_Asp2512del and p.Val3681_Asn3686del) were included in the study. The mutations have been identified in patients with NM or distal myopathy. We have performed F-actin and tropomyosin binding experiments for NEB fragments using co-sedimentation and GST-pull-down assays. NEB fragments containing the p.Glu2431Lys mutation and protein fragments lacking the entire exon 55showed reduced affinity to F-actin compared with wt fragments. Protein fragments containing the p.Ser6366Ile mutation at an actin-binding site showed increased actin affinity. Protein fragments containing the p.Glu2431Lys mutation showed higher and fragments containing the p.Thr7382Pro mutation showed lower affinity to tropomyosin than wt proteins. Our results demonstrate actin-nebulin interactions and for the first time tropomyosin-nebulin interactions in vitro, and show that the interactions are altered by mutations.

V.P12.

Exercise modifies titin phosphorylation and reduces cardiac myofilament stiffness

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The giant sarcomeric protein titin is a major contributor to passive stiffness in striated muscle tissue. Cardiac titin stiffness is largely determined by the composition of the titin isoforms N2BA (~3.2–3.7 MDa, compliant) and N2B (~3.0 MDa, stiffer). More acutely, titin stiffness is modified by domain-specific phosphorylation. Interestingly, phosphorylation of the N2-B unique sequence (N2-Bus) by PKA, PKG or CaMKII decreases titin stiffness, whereas phosphorylation of the PEVK region by PKCalpha increases it.

Exercise is a potent modifier of cardiac kinase activity, and has been shown to improve myofilament function, e.g. after myocardial infarction. Here, we test the hypothesis that exercise acutely changes titin phosphorylation in the heart and skeletal muscle and thereby modifies titin-based myofilament stiffness.

Adult Sprague-Dawley rats were exercised on a treadmill for 15 min performing downhill (−20°) running. Titin phosphorylation was determined by Western blot analysis using phosphospecific antibodies to Ser383 in the N2-Bus region, and to Ser11878 in the PEVK region. Exercise did not change N2-Bus phosphorylation at Ser282 but significantly decreased the phosphorylation status of Ser11878 in the PEVK-region by over 50 % in cardiac tissue and more than 95 % in skeletal biopsies. Passive tension (PT) was determined on isolated skinned cardiomyocytes, and was decreased by up to 30 % in cardiomyocytes from exercised animals compared to the control group. In summary, acute exercise induces rapid changes in the titin phosphorylation status that decrease cardiac titin stiffness, and may therefore improve diastolic function during physical exercise.

V.P13.

Xin repeat proteins interact with SH3 domains of nebulin and nebulette during myofibril formation and remodeling

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The Xin actin-binding repeat-containing proteins Xin and XIRP2 are exclusively expressed in striated muscle cells, where they are supposed to play an important role in development. In adult muscle, both proteins are concentrated in myotendinous junctions and intercalated discs, the attachment sites of the myofibrils to the membrane. In contrast, during development they are localized to immature non-striated premyofibrils together with their binding partner filamin C, indicating an involvement of both proteins in myofibril assembly. We have identified the SH3 domains of nebulin and nebulette as novel ligands of specific proline-rich regions of Xin and XIRP2. The precise binding motifs were mapped, and shown to bind both SH3 domains with micromolar affinity. Co-crystallization of the nebulette SH3 domain with the interacting XIRP2 peptide PPPTLPKPKLPKH revealed selective interactions that conform to class II SH3-domain-binding peptides. BiFC experiments in cultured skeletal muscle cells and primary cardiomyocytes indicated a temporally restricted interaction of both Xin repeat proteins with nebulin and nebulette during early stages of myofibril development that is lost upon further maturation. In highly developed striated muscle cells, this interaction is limited to longitudinally oriented structures that are associated with myofibril development and remodeling. These data provide new insights in the role of Xin-repeat proteins together with their newly and previously identified interaction partners in myofibril assembly and remodeling after muscle damage.

V.P14.

A mutation in NEB induces severe muscle weakness in a mouse model for nemaline myopathy

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Nebulin plays a pivotal role in skeletal muscle contractility by specifying thin filament length and function. Although mutations in the gene encoding nebulin (*NEB*) are a frequent cause of nemaline myopathy (NM), the most common non-dystrophic congenital myopathy, the mechanisms by which mutations in *NEB* cause muscle weakness remain largely unknown.

To better understand these mechanisms, we have generated a mouse model in which *Neb* exon 55 is deleted ($Neb^{\Delta ex55}$) to replicate a founder mutation seen frequently in NM patients with Ashkenazi Jewish heritage.

$Neb^{\Delta ex55}$ mice are born close to Mendelian ratios, but show growth retardation after birth. Electron microscopy studies show nemaline bodies—a hallmark feature of NM—in muscle fibers from $Neb^{\Delta ex55}$ mice. Western blotting studies with nebulin-specific antibodies reveal reduced nebulin levels in muscle from $Neb^{\Delta Exon55}$ mice, and immunofluorescence confocal microscopy studies with tropomodulin antibodies and phalloidin reveal that thin filament length is significantly reduced. In line with reduced thin filament length, the maximal force generating capacity of permeabilized muscle fibers and single myofibrils is reduced in $Neb^{\Delta ex55}$ mice with a more pronounced reduction at longer sarcomere lengths. Finally, in $Neb^{\Delta ex55}$ mice the regulation of contraction is impaired, as evidenced by marked changes in cross bridge cycling kinetics and by a reduction of the calcium sensitivity of force generation. A novel drug that facilitates calcium binding to the thin filament significantly augmented the calcium sensitivity of submaximal force to levels that exceed those observed in untreated control muscle.

We have characterized the first nebulin-based NM model, which recapitulates important features of the phenotype observed in patients harboring this particular mutation, and which has severe muscle weakness caused by thin filament dysfunction.

V.P16.

Characterization of CAPN3-dependent proteolysis of C-terminal titin

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The titinopathies tibial muscular dystrophy (TMD) and limb-girdle muscular dystrophy 2J (LGMD2J) are caused by mutations in C-terminal titin, residing in the sarcomeric M-band. Mutations identified so far affect the last Ig domain M10 or the preceding is7 region. The FINmaj mutation, underlying TMD/LGMD2J in patients of Finnish origin, changes four amino acids in M10 and presumably leads to domain unfolding. The other known mutations cause missense changes of single amino acids or truncation of the ultimate C-terminus. Loss of C-terminal titin epitopes in immunofluorescence, and reduced amount of C-terminal titin fragments in western blotting suggest that increased or abnormal proteolytic turnover of mutant titin may contribute to the pathomechanism. The protease calpain 3 (CAPN3) binds M-band titin at the is7 region; the interaction is thought to regulate its autolytic activation. LGMD2J patients and FINmaj knock-in mice show secondary CAPN3 deficiency, reflecting loss of the binding site and consequent dysregulation of CAPN3 activity.

To elucidate the proteolytic events involved in the pathogenesis of M-band titinopathies, we investigated the cleavage of C-terminal titin by CAPN3. Cleavage fragments generated by CAPN3 were identified by coexpressing various wild-type and mutant titin constructs with active or inactive CAPN3 in cell culture, and comparing the resulting fragment patterns. Active CAPN3 produced several titin fragments, which were characterized by western blotting, protein sequencing and mass spectrometry for identification of cleavage sites. Targeted mutagenesis of predicted CAPN3 recognition sites was utilized for understanding the sequence determinants of CAPN3 cleavage and studying the order of cleavage events.

V.P17.

The nebulin family protein Lasp regulates thin filament length in myofibrils

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Lasp is the only member of the nebulin family in *Drosophila*, and, like its vertebrate ortholog Lasp-1, consists of a LIM domain followed by two nebulin repeats and a C-terminal SH3 domain. Vertebrates have four additional nebulin family members, the namesake nebulin, nebulette, NRAP, and Lasp-2. Here we demonstrate that Lasp carries out all the functions taken over by nebulin in vertebrates. In myofibrils Lasp localizes to the Z-disc and the A-band. We can show that *Lasp* mutants have shorter thin filaments and wider Z-discs, and in consequence, reduced crawling and flying ability. These phenotypes were observed across all muscle types analyzed (body wall muscles, leg muscles, and indirect flight muscles). We also observe phenotypes hitherto undescribed in vertebrate nebulin mutants, in particular a requirement of Lasp for proper thick and thin filament spacing in myofibrils. Finally, we identified interaction partners of Lasp through affinity purification and mass spectrometry, and we provide evidence via FRAP experiments that Lasp is a peripheral regulatory component of the Z-disc rather than a scaffold protein.

V.P18.

Ultrastructural localisation of titin during sarcomere assembly in regenerating vertebrate skeletal muscle after intoxication with notexin

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The giant filamentous protein titin spans from the Z-disk to the M-line in the sarcomere of striated muscle cells, where it functions as a molecular spring during stretching and relaxation. During myofibrillogenesis, titin is thought to act as a molecular template for the assembly of A-bands, because super-repeats of titin domains in the C-zone of the A-band show a $\sim$ 43 nm periodicity coinciding with the $\sim$43 nm spacing of myosin heads and MyBP-C.

In this study we used immunogold electron microscopy and three-dimensional reconstruction of electron micrographs to localise titin, α-actinin, myosin, myosin binding protein-C and myomesin at different stages of myofibrillogenesis in regenerating rat *soleus* muscle after notexin-induced myofibre breakdown. 2 days after intoxication with notexin we observed premyofibrils containing titin, myosin and Z-bodies; 3 days after intoxication we found Z-bodies fusing to

Z-disks and I- and A-Bands beginning to show typical striation patterns. Three dimensional reconstructions revealed that extrasarcomeric titin colocalises with thick filaments in sarcomeres and is also nearby the assembling sarcomeres. Our results support a model in which titin acts as a molecular scaffold protein for the assembly and the integration of the thick filaments into the sarcomere during skeletal muscle regeneration.

V.P19.

The type of chronic in vivo mechanical loading of rat skeletal muscles induces alterations of extracellular matrix structural constituents

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The extracellular matrix (ECM) characterizes dense scaffolds of broad diversities of protein classes and thus provides mechanical stability to tissues, including skeletal muscle. The local molecular composition of the ECM determines structural and mechanical characteristics and thus regulates skeletal muscle integrity. Local fibrillar collagen types I and III (Col1a1/a3) as well integrin alpha7 (Itga7) are central constituents for skeletal muscle adaptations towards loading. However, only limited knowledge exists on effects of long-term in vivo loading and muscle contraction types on both mRNA and protein profiles. We hypothesized that different muscle contraction types cause distinct adaptations of fibrillar Co1a1/a3, Itga7 and collagen-maturating enzymes prolyl 4-hydroxylases (P4ha1/2) towards in vivo long-term loading.
32 Sprague-Dawley rats were assigned to one of following groups: control (CON, sedentary), level (concentric contractions, 0° decline) or downhill (eccentric contractions, −20° decline) loading (each exercise lasted 6 weeks). RNA and tissue homogenates were prepared from both medial gastrocnemius (MG) and quadriceps femoris (QUF).
No changes were observed in both muscles for Col1a1/a3 as well as P4ha1/2 mRNAs. Itga7 increased in QUF muscle after both interventions. Importantly, Col1a1/a3 proteins were critically dependent on both muscle and contraction type as were P4ha1/2 and Itga7.
Our data show that chronic concentric and eccentric in vivo loading distinctly determine the local ECM microenvironment. Therefore, our results are critical for the understanding of skeletal muscle ECM adaptations towards physiological loading models as used in exercise-based medical therapies and they uncover the biological significance of exercise to establish new local muscle ECM equilibriums.

POSTER SESSION VI Diaphragm physiology

VI.P1.

Sparing of muscle mass and function by passive loading in an experimental intensive care unit model

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The mechanical silencing observed in intensive care unit (ICU) patients leads to a severe and specific muscle wasting condition. The response to mechanical stimuli is an important factor in the regulation of cell physiological and pathophysiological function. This study aims to unravel the underlying mechanisms and the effects of passive mechanical loading on skeletal muscle mass and function. Specific interest is focused on gene/protein expression and intracellular signaling. A unique experimental rat ICU model has been used allowing time-resolved analyses of the effects of standardized unilateral passive mechanical loading on skeletal muscle size, function and underlying mechanisms. Results show that passive mechanical loading alleviated the muscle wasting and the loss of force-generation associated with the ICU intervention, resulting in a doubling of the functional capacity of the loaded versus the unloaded muscles after a 2-week ICU intervention. We demonstrate that the improved maintenance of muscle mass and function is probably a consequence of a reduced oxidative stress revealed by lower levels of carbonylated proteins, and a reduced loss of the molecular motor protein myosin. We investigated if the improvement also could be seen on proteins in the mTORC1 signaling pathways, but no difference was seen between the loaded versus the unloaded muscle, suggesting that other pathways are involved. Thus, the results from this study show that passive mechanical loading alleviates the severe negative consequences on muscle size and function associated with the mechanical silencing in ICU patients, strongly supporting early and intense physical therapy in immobilized ICU patients.

VI.P2.

Diaphragm contractile dysfunction in heart failure: role of the p47^{PHOX} subunit of NADPH oxidase

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Patients with chronic heart failure (CHF) suffer from shortness of breath, weakness, and fatigue. These symptoms are determined largely by respiratory muscle (mainly diaphragm) weakness. Several studies have shown that CHF causes diaphragm contractile dysfunction. However, the specific pathways and mechanisms leading to diaphragm weakness are less clear. Oxidants are increased in CHF diaphragm and independent studies have shown that oxidants impair muscle contractile properties. NADPH oxidase is a predominant source of oxidants in skeletal muscle, and may be responsible for diaphragm weakness in CHF. The NOX2 isoform of NADPH oxidase consists of several subunits, with p47^{phox} being required for enzyme activation. Therefore, we examined the role of p47^{phox} subunit of NADPH oxidase on diaphragm weakness in heart failure. We induced myocardial infarction in mice deficient in p47^{phox} and wild-type genetic controls (WT) and performed experiments 12–16 weeks post-surgery. Controls underwent sham surgery (Sham). Left ventricular fractional shortening measured by echocardiography was $\sim$ 45 % lower ($p < 0.05$) after myocardial infarction, which is consistent with the development of CHF. CHF decreased diaphragm maximal tetanic force in WT mice by $\sim$25 % ($p < 0.05$; n = 8–9/group). However, diaphragm maximal tetanic force was not different in Sham and CHF p47^{phox} knockout mice ($p > 0.05$; n = 7–9/group). We also observed that CHF impaired diaphragm isotonic contractile properties in WT but not in p47^{phox} knockout mice. Overall, our data suggest that the p47^{phox} subunit of NADPH oxidase is required for diaphragm contractile dysfunction in CHF mice.

VI.P4.

Diaphragm muscle weakness in a novel mouse model for nebulin-based nemaline myopathy

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Background Nemaline myopathy (NM) is the most common non-dystrophic congenital myopathy, and is characterized by muscle weakness. Seven genes—all coding thin filament proteins—are implicated in NM. The main form of NM results from nebulin gene (*NEB*) mutations. Recently, we have generated a mouse model with a deletion of *NEB* exon 55, a mutation known to frequently occur in NM patients.
Importantly, this model allows studying the diaphragm, a muscle whose function is very difficult to investigate in human NM. It has been postulated that the diaphragm is particularly affected in NM, and that this explains why many children with NM develop respiratory failure. Here we used $Neb^{\Delta Exon55}$ mice to test the hypothesis that diaphragm strength is more affected than peripheral muscle strength.
Methods Small (diameter $\sim$70 μm) fiber bundles of m. diaphragm and soleus were dissected from homozygous $Neb^{\Delta Exon55}$ mice and from wt littermates. Bundles were permeabilized and mounted between a force transducer and length motor. Sarcomere length was set at 2.3 μm. Fiber bundles were activated by exposure to solutions with saturating [Ca^{2+}] (pCa 4.5) and non-saturating [Ca^{2+}] (pCa 5.8). Maximal tension, crossbridge cycling kinetics (*ktr*), active stiffness and calcium-sensitivity of force were determined.
Results Data are summarized in the table below.

	Diaphragm $Neb^{\Delta Exon55}$ (fraction of wt)	Soleus $Neb^{\Delta Exon55}$ (fraction of wt)
Maximal Tension (pCa 4.5; mN/mm^2)	0.27 ± 0.03*,#	0.44 ± 0.02*
Ktr (pCa 4.5; s^{-1})	0.53 ± 0.03*,#	0.79 ± 0.02*
Active Stiffness (pCa 4.5; mN/mm^2)	0.25 ± 0.03*	0.33 ± 0.01*
Calcium Sensitivity (Force at pCa 5.8/4.5)	0.74 ± 0.02*,#	0.85 ± 0.04*

Mean ± SEM; * $p < 0.05$ WT vs. $Neb^{\Delta Exon55}$; # $p < 0.05$ Diaphragm $Neb^{\Delta Exon55}$ vs. Soleus $Neb^{\Delta Exon55}$

Conclusion In line with our hypothesis we observed that the diaphragm muscle is more affected than peripheral skeletal muscle in this mouse model of nebulin-based NM. We speculate that preferential diaphragm weakness contributes to respiratory failure in NM.

VI.P5.

Diaphragm degeneration aggravates cardiac muscle injury in dystrophin-deficient mouse

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We examined whether diaphragm degeneration affects cardiac muscle in dystrophin-deficient mdx mice. To worsen diaphragm degeneration, adult mdx mice were exercised (swim) for 1 h, 5 days a week, for 2 months. One day after the last exercise, diaphragm and hearts were analyzed by histopathology and western blot of metalloproteinase (MMP) and tumor growth factor (TGF)-beta. Age matched unexercised mdx served as controls. In mdx diaphragm, exercise increased fibrosis (60 %) and the levels of active MMP-2, pre MMP-2 and pro MMP-2 (26, 28 and 12 % increase). In mdx heart, exercise also lead to increased fibrosis (42 % higher than unexercised), higher levels of TGF-b and of active MMP-2, pre MMP-2 and pro MMP-2 (32, 28 and 11 % increase) compared with unexercised mdx. Density of inflammatory cells patches was almost twice higher in exercised than unexercised mdx hearts. Right ventricle was significantly more affected than the left ventricle: myocardial fibrosis was increased by 45 % (8.3 ± 0.6 in unexercised vs. 12.0 ± 0.6 in exercised) in the right ventricle while in the left ventricle this increase was 35 % (5.6 ± 0.7 unexercised vs. 7.6 ± 0.8 exercised). Inflammatory cells/degenerating cardiomyocytes density was increased 95 % (2.3 ± 0.6 unexercised vs. 4.5 ± 0.8 in exercised) in the right ventricle against 71 % increase in left ventricle (1.4 ± 0.6 unexercised vs. 2.4 ± 0.5 exercised). While exercise promoted concomitant changes in the dystrophic diaphragm and heart, the present results suggest that degeneration of diaphragm may contribute, at least in part, to the cardiac injury in dystrophin deficient mice.

VI.P6.

Leaky ryanodine receptors contribute to diaphragmatic muscle weakness during mechanical ventilation

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Ventilator-induced diaphragmatic dysfunction (VIDD) refers to the diaphragm muscle weakness that follows prolonged controlled mechanical ventilation (MV). The presence of VIDD impedes recovery from respiratory failure, but the pathophysiological mechanisms accounting for VIDD are still not fully understood. Here we show in human subjects as well as animal models of VIDD (pigs and mice) that MV is associated with rapid remodeling of the sarcoplasmic reticulum (SR) calcium release channel/ryanodine receptor (RyR1) in the diaphragm. The RyR1 macromolecular complex was oxidized, S-nitrosylated and Ser-2844 phosphorylated and depleted of the stabilizing subunit calstabin1, following MV. These post-translational modifications of RyR1 were mediated by oxidative stress and resulted in abnormal resting SR Ca^{2+}-leak and reduced contractile function. Treatment with S107, a small molecule drug that stabilizes the RyR1-calstabin1 interaction despite these RyR1 complex modifications effectively prevented VIDD. Diaphragm dysfunction is common in MV patients and is a major cause of failure to wean patients from artificial respiratory support. The present study provides the first evidence of RyR1 alterations as a proximal mechanism underlying VIDD as well as a potential therapeutic target.

VI.P7.

Nitric oxide modifies force production and fatigue response in diaphragm strips from *mdx* mice

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Duchenne muscular dystrophy (DMD) affects young boys and is characterized by the absence of dystrophin, a large cytoskeletal protein present in skeletal and cardiac muscle cells. The heart and diaphragm become necrotic in DMD patients and animal models of DMD, resulting in cardiorespiratory failure as the leading cause of death. The major consequences of the absence of dystrophin are high levels of intracellular Ca^{2+} and the unbalanced production of NO. The absence of dystrophin disturbed Ca^{2+}-homeostasis and reduces the activity of the neuronal isoform of nitric oxide synthase (nNOS), which is bound to dystrophin via syntrophin. The lack of NO synthesis from nNOS triggers the deregulation of several NO-dependent pathways, such as Ca^{2+}-release, metabolism, immune, and vascular responses. However, there is no evidence how these two second messengers would interact during diaphragm activation in absence of dystrophin. Using strips from the mouse hemi-diaphragm, we studied the force production and fatigue response to electrical stimulation in presence of different pharmacological tools able to modify Ca^{2+}-release and re-uptake together with NO donors or NOS blockers. Here, we present that NO in fact alters the force production (1–180 Hz, 2 ms, 50 V) and fatigue response (3 and 60 Hz, 2 ms, 50 V) in WT murine's strips of diaphragm, probably modifying Ca^{2+}-homeostasis. This action is more severe in murine DMD animal model, where force and fatigue response are largely modified. Our results suggested that diaphragmatic force production Ca^{2+}-dependent is modulated by the NO generated by nNOS.

VI.P8.

Combined effects of Controlled Mechanical Ventilation (CMV) and aging on rat diaphragm muscle single fibers

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We have studied the combined effects of Controlled Mechanical Ventilation (CMV) and aging on diaphragm muscle single fibers using a unique rat model of Intensive Care Unit (ICU) conditions. Two populations of Brown Norway Fisher/433 rats were considered: Young (7–8 months) and Old (27–29 months) and further grouped in: (1) "Young controls" (n = 2); (2) "Old controls" (n = 2); (3) "Young 5 days" (n = 3); (4) "Old 5 days" n = 3; the "-5 days" groups were deeply sedated with isoflurane, paralyzed with α-cobratoxin, hydrated and fed intravenously, mechanically ventilated through oro-tracheal tube. After 5 days the mid-costal parts of the diaphragm were dissected out, divided into bundles and membrane-permeabilized in skinning solution. The Cross Sectional Area (CSA) of the single fibers, and the Specific Force (absolute force/CSA) were measured.

Results (1) the fibers' CSA from "Young controls" was significantly lower compared with "Old controls"; (2) vice versa, the specific force was significantly higher in "Young controls" Vs "Old controls"; (3) the CSA increased in both "Young 5 days" and "Old 5 days" rats, but (4) the difference between the 2 groups was attenuated; at the contrary (5) the specific force decreased after 5 days in both Young and Old, but 6) the difference Young Vs Old declined.

Conclusions (a) after 5 days of deep sedation and CMV the rat diaphragm muscle fibers were hypertrophic but weaker; (b) the differences of Young Vs Old in CSA and specific force decreased as well.

POSTER SESSION VII Muscular dystrophies and congenital myopathies

VII.P1.

Myocyte contractile function in tetralogy of fallot

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Hypertrophic cardiomyopathy (HCM) due to sarcomeric gene mutations is characterised by left ventricular hypertrophy and cardiac myocyte disarray. We found previously that myocytes from HCM patients show no change in passive stiffness (compared with healthy donors) but reduced active force, faster crossbridge kinetics and enhanced Ca^{2+}-sensitivity. To investigate whether similar changes are seen in hypertrophy resulting from pressure overload rather than gene mutations, we compared the properties of myocytes taken from the right ventricle of children with Tetralogy of Fallot (ToF, age 4–17 months) with myocytes taken from healthy (unused donor) hearts (age 19–54 year; age-matched donor controls were unavailable). Skinned myocytes were prepared from ToF and donor tissue. Maximal Ca^{2+}-activated force was lower in ToF myocytes (24 ± 2kN/m^2; mean ± SE, n = 8 patients) than in donor myocytes (35 ± 3 kN/m^2, n = 6; p = 0.01) but Ca^{2+}-sensitivity was not significantly different (ToF: $pCa_{50} = 6.09 \pm 0.05$; donor: $pCa_{50} = 6.02 \pm 0.03$). The rate of force re-development after a stretch/release protocol (k_{tr}) during maximal Ca^{2+}-activation was significantly higher in ToF myocytes than in donor myocytes (2.46 ± 0.08 s^{-1} vs 2.05 ± 0.10 s^{-1}, respectively; p = 0.02). Passive stiffness was measured by applying a series of step sarcomere length stretches and was found to be similar in ToF and donor myocytes. In conclusion, ToF myocytes, like HCM myocytes, showed reduced force and faster crossbridge kinetics during maximum activation and no change in passive stiffness but, unlike HCM myocytes, ToF myocytes showed similar myofilament Ca^{2+}-sensitivity to donor myocytes. The differences in maximum Ca^{2+}-activated force and kinetics may reflect features common to hypertrophic myocardium.

VII.P2.

Cardiopulmonary exercise testing in ambulatory children with Duchenne and Becker Muscular Dystrophy

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Introduction The cardiopulmonary exercise test (CPET) could be a relevant outcome measure for exercise capacity in children with muscular dystrophy (MD) but knowledge of exercise response and safety is scarce.

Methods The CPET was assessed in nine children with Duchenne (n = 3) and Becker (n = 6) Muscular Dystrophy. Feasibility parameters and exercise response were evaluated and compared to a standard care test protocol including the six-minute walk test (6MWT).
Results Nine boys (age 10.8 ± 4.7) with DMD (n = 3) and BMD (n = 6) participated in this study. Mean exercise duration was 8.3 ± 3.6 min. Peak heart rate ranged from 126 to 195 (mean 156 ± 23), while RER_{peak} ranged from 0.88 to 1.31 (mean 1.1 ± 0.18). Exercise capacity (peak VO_2) was normal (>82 %) in one child, mildly reduced (61–82 %) in three and moderately to severely reduced (<60 %) in five children, whereas these same numbers for 6MWT were respectively three, four and two. There were no signs of rhabdomyolysis, cardiac events, inadequate blood pressure or unacceptable levels of muscle pain. Acceptability for CPET was higher in older and/or less impaired children.
Conclusion In children with muscular dystrophy and relative mild impairments CPET seems to be a feasible and safe outcome measure. Outcomes show adequate RER_{peak}, but low HR_{peak}, which may be a sign of inadequate chronotrope activity of the cardiac system. CPET showed more severe limitations compared to reference values than the 6MWT and may be more sensitive for changes in the early stages of disease or in less impaired children.

VII.P3.

Skeletal muscle myopathy mutations in tropomyosin gene TPM3 affect thin filament transitions between the inactive and active states

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It has recently been discovered that nemaline myopathy and cap myopathy can be caused by mutations in β-tropomyosin (*TPM2* gene) and that a third skeletal muscle myopathy, congenital fibre-type disproportion, is predominantly due to mutations in slow α-tropomyosin (*TPM3* gene). We have expressed tropomyosin TPM3 mutants R90P, R167 G/C and R244G in a baculovirus-Sf9 insect cells system and studied reconstituted actin-tropomyiosin mutant filaments biochemical properties. We analysed the effects of these TPM3 mutations on the biochemical properties of tropomyosin namely actin binding and the equilibrium constants of transitions between the 'open', 'closed' and 'blocked' states of thin filament and the cooperativity of these transitions. We also assessed their functional effects on the actomyosin ATPase. We found that TPM3 mutations affected troponin-tropomyosin inhibition and activation of actomyosin ATPase. TPM3 mutations also affected the Blocked-closed and closed-open transitions. These findings are in agreement with recent structural studies of tropomyosin bound to actin in the closed or blocked conformations. We propose that destabilisation of the actin-tropomyosin interface by charge change in the closed or blocked state may represent the underlying biochemical defect for the onset of skeletal muscle myopathies.

VII.P4.

Multimodal MRI and ^{31}P-MRS investigations of the *ACTA1* (Asp286Gly) mouse model of nemaline myopathy provide evidence of impaired in vivo muscle function, altered muscle structure and disturbed energy metabolism

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Introduction Nemaline myopathy (NM), the most common of the non-dystrophic congenital myopathies, is characterized by muscle weakness. Mutations in the ACTA1 gene have been found in the most severely affected cases (>50 %). A transgenic mouse model carrying the human Asp286Gly mutation has been recently generated and reproduced the main clinical and pathological features of NM patients. In this study, we reported the first in vivo anatomical, functional and metabolic characterization of the Asp286Gly mouse model.
Methods Maximal tetanic force production (at 150 Hz), hindlimb muscles volume and microstructure using multimodal magnetic resonance (MR) imaging (T2 and Diffusion Tensor Imaging (DTI)) and energy metabolism during a fatiguing protocol using 31-phosphorus MR spectroscopy were assessed in 4 month-old Asp286Gly and control mice.
Results Maximal absolute force production (−30 %) and muscles volume (−15 %) were reduced in Asp286Gly mice leading to a lower specific force production (−15 %). T2 values were increased in Asp286Gly mice and linearly related to muscle weakness. DTI parameters were reduced and the energy cost of contraction was increased in Asp286Gly mice.
Conclusion In Asp286Gly mice, muscle weakness was partially related to muscle atrophy. Accordingly, the higher energy cost might be ascribed to an alteration occurring at the cross-bridge level. Increased T2 values and alterations of DTI parameters might be related to the occurrence of muscle degeneration/regeneration process and/or the presence of histological anomalies. Overall, we reported structural and biochemical defects in the Asp286Gly model. The corresponding variables might be used as diagnostic biomarkers for NM and prognostic indices for therapeutic interventions.

VII.P5.

Near-infrared spectroscopy during exercise and recovery in children with juvenile dermatomyositis

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Background It is hypothesized that microvascular disturbances in muscle tissue play a role in the reduced exercise capacity in juvenile dermatomyositis (JDM).
Methods Children with JDM, children with juvenile idiopathic arthritis (clinical controls), and healthy children performed a maximal incremental cycloergometric test from which normalized concentration changes in oxygenated hemoglobin ($\Delta[O_2Hb]$) and total hemoglobin (Δ[tHb]) as well as the half recovery times of both signals were determined from the vastus medialis and vastus lateralis using near-infrared spectroscopy.
Results Children with JDM had lower Δ[tHb] values in the vastus medialis at work rates of 25, 50, 75, and 100 % of maximal work rate compared with healthy children; the increase in Δ[tHb] with increasing intensity seen in healthy children was absent in children with JDM. Other outcome measures differed not by group.
Discussion The results suggest that children with JDM may experience difficulties in increasing muscle blood volume with more strenuous exercise.

VII.P6.

Neurotrophic factors in the limb and the extraocular muscles of mouse model of ALS

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Amyotrophic lateral sclerosis (ALS) is a fatal, neurodegenerative disease affecting upper and lower motor neurons, causing progressive muscle paralysis. While practically all skeletal muscles are affected, extraocular muscles (EOMs) are relatively spared in ALS. Neurotrophic factors (NTFs) promote survival and maintenance of specific populations of neurons and regulate neuronal differentiation. We investigated the mRNA levels and tissue distribution of BDNF, NT3, NT4 and GDNF in EOMs and limb muscles of $SOD1^{G93A}$ transgenic mice at different stages of ALS using qRT-PCR and immunohistochemistry. Significantly increased GDNF mRNA level was found in EOMs at early stage and in limb muscles at terminal stage whereas significantly decreased mRNA level of NT4 was found in EOMs at early stages. NTF was detected in a variety of structures in the muscle sections, including nerve axons, neuromuscular junctions (NMJs), myotendinous junctions, nuclei and muscle fibers. The alterations of NTFs were inconsistent among different structures. The expression of the four NTFs was down regulated at NMJs in limb muscles but not at NMJs in EOMs, which may reflect the distinct capacity of the EOMs to maintain NMJs. The discrepancy of alterations among different structures in both limb and EOMs may reflect distinct impact of ALS on these structures, and explain the heterogeneity of mRNA levels of the NTFs.

VII.P7.

Serum matrix metalloproteinase-9 (MMP9) as a biomarker for monitoring Duchenne Muscular Dystrophy (DMD) disease progression

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The identification of non-invasive biomarkers in Duchenne muscular dystrophy (DMD) patients is one of the unmet needs in clinical practice. The aim of this study was to identify a serum biomarker able to monitor disease progression and response to treatment in DMD patients starting from candidate markers which have been associated in the past with DMD pathology.

We used commercially available ELISA assays to evaluate serum levels of several markers in DMD patients and compared these to age-matched controls. Matrix Metalloproteinase-9 (MMP9) was the most promising candidate. Levels of MMP9 were measured in 211 serum samples obtained from 120 DMD patients followed up in Newcastle, Leiden and London. MMP9 levels were higher in DMD patients compared to age-matched healthy controls. Elevated MMP9 levels were confirmed by gelatin zymography, showing the strongest correlation between ELISA results and the inactive MMP9 form. Longitudinal data from 71 DMD patients followed up in Leiden and Newcastle for over 5 years showed that MMP9 significantly increased over time. Hence, MMP9 is a good candidate biomarker to monitor disease progression in DMD patients. These data are supportive to evaluate the potential of MMP9 as a biomarker to determine the early response to treatments.

VII.P8.

Antisense oligonucleotide-mediated knockdown of TGF-β/myostatin type I receptors as a potential therapy for duchenne and other muscular dystrophies

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Skeletal muscle fibrosis and impaired muscle regeneration are major factors that contribute to progressive decline of muscle function in Duchenne Muscular Dystrophy (DMD) and other types of muscle dystrophies. TGF-β/myostatin signaling is directly involved in DMD pathology and inhibition of these signaling cascades has been shown to improve regeneration and reduce fibrosis in the dystrophic muscle. We developed an efficient method to selectively inhibit the function of type I TGF-β receptors Acvr1b (ALK4) and Tgfbr1 (ALK5) based on antisense oligonucleotide (AON)-mediated exon skipping. Our results show that both myostatin type I receptor ALK4 and myostatin/TGF-β type I receptor ALK5 can be efficiently downregulated in vitro. AON-mediated exon skipping in ALK4 and ALK5 receptors resulted in increased myogenic differentiation of C2C12 myoblasts. In addition, efficient AON-mediated knockdown of ALK4 and ALK5 was achieved in vivo after intramuscular and systematic injection in *mdx* mice, a DMD mouse model. To summarize, our experiments suggest that this novel strategy of AON-mediated targeting of myostatin/TGF-β receptors may provide a therapy to selectively inhibit myostatin and TGF-β signaling and improve DMD muscle quality and function. Further studies will investigate the short- and long-term effect of AON treatment in the DMD mouse model and other myopathic mouse models.

VII.P9.

Expression of ADAMTS5 in mdx mice throughout pathology

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Satellite cells play a central role in skeletal muscle regeneration and the importance of the satellite cell niche, which surrounds them, is starting to evolve. A critical component of the niche environment is the composition and processing of the extracellular matrix. There are a large array of proteases which regulate the composition of the extracellular matrix that have been implicated in development,

growth and regeneration. ADAMTS5, an extracellular metalloproteinase, predominantly remodels proteoglycans in the basal lamina such as versican, biglycan and decorin. ADAMTS5 *gene* expression is regulated in the progression of pathology in the mdx mouse model of Duchenne Muscular Dystrophy (DMD), implicating ADAMTS5 in DMD pathophysiology. Recent work has shown that ADAMTS5 and versican are expressed in developing skeletal muscle and that inhibiting ADAMTS5 activity adversely affects in vitro myotube formation. Versican remodeling, detected using a neo-epitope antibody that detects an ADAMTS-specific cleavage product of versican, was shown to be associated with these effects on myoblast fusion. In an extension to these in vitro studies we are investigating the cell types which express ADAMTS5 in regenerating and dystrophic skeletal muscle. Initial immunohistochemical staining suggests that ADAMTS5 protein is associated with mononucleated cells at the periphery of muscle fibres and also newly regenerated, embryonic myosin positive myofibres.

VII.P10.

SERCA1b expression in human neonatal muscles and human dystrophic muscle diseases

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The sarcoplasmic reticulum calcium ATPase, SERCA1 has two isoforms: SERCA1a (in adult fast fibres) and SERCA1b (in neonatal/regenerating myotubes). The two isoforms are created by alternative splicing. The penultimate exon containing the first translation stop is skipped in the SERCA1b mRNA and a DEDPERRK C-terminal is translated instead of a single glycin of the SERCA1a protein. Using antibody against the C-terminal octapeptide tail and a pan SERCA1 antibody, our group previously described that SERCA1b was expressed only in regenerating muscle and muscle cell culture. Muscle regeneration recapitulates neonatal development therefore we investigated SERCA1b protein expression in post mortem human neonatal muscles. We also examined myotonic dystrophy type 2 and Duchenne muscular dystrophy since these conditions are accompanied with impaired SERCA1 splicing and/or regeneration process in their pathomechanism. Fetal and neonatal samples were taken post mortem from 4 different muscles of 5 subjects (including intrauterine exits to 8 weeks old newborns), for pathologic muscles biopsies were investigated. Sarcoplasmic protein fractions were used for Western blotting, and immunohistochemistry was done on frozen sections.

SERCA1b protein was expressed in an age, muscle and disease specific manner according to Western blots. In contrast to regenerating rat soleus sections, where all fibres showed intense positivity for SERCA1b, in neonatal human muscles only a few fibres stained with SERCA1b but not with the panSERCA1 antibody revealing uncertainty of the immunohistochemical detection of the neonatal isoform. We conclude that human late foetal, neonatal muscles and in certain illnesses even adult muscles express SERCA1b which can be unambiguously revealed by immunoblot and not by immunohistochemistry.

VII.P11.

Single muscle fibre mechanical studies give insight into the molecular mechanism of muscle weakness in patients with tropomyosin myopathy

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Tropomyosin (TM) is an important regulatory protein of the skeletal muscle sarcomere. Together with the troponin complex, TM regulates the calcium-dependent formation of cross-bridges between the myofilaments. Mutations in TPM3 (encoding slow alpha TM slow) cause congenital myopathy characterised by generalized skeletal muscle weakness. In previous studies, an N-terminal mutation in TM was shown to affect TM dimerisation, resulting in abnormal isoform composition, which was proposed to cause muscle dysfunction in these patients. However, the disease mechanism may vary depending on the mutation.

In this study we aim to understand muscle dysfunction in 10 patients with 6 dominant *TPM3* mutations. We determined if changes in TM isoform composition are a common cause of muscle weakness. In addition, we studied the effect of *TPM3* mutations on the sarcomere by performing a range of contractile measurements on single muscle fibres from patient biopsies.

We showed that TM isoform composition is normal in all patients tested. Contractile measurements showed reduced maximal force generation, reduced cross-bridge cycling kinetics and reduced calcium sensitivity exclusively in slow muscle fibres, where the slow TM isoform is expressed.

Our results demonstrate that TM isoform composition is not commonly altered in *TPM3*-related myopathy. Most mutations appear to directly affect cross-bridge cycling and calcium sensitivity of the thin filament perhaps by changing TM's interaction with the actin filament. Recently, a number of drugs have been developed that specifically target calcium sensitivity of muscle contraction. Our findings indicate that they may improve muscle strength in many patients with TM-related congenital myopathy.

VII.P12.

Muscle dysfunction and structural defects of dystrophin-null *sapje* mutant zebrafish larvae are rescued by ataluren treatment

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Background *Sapje* zebrafish carries a mutation in the dystrophin gene resulting in a premature stop-codon, and a severe muscle phenotype. They display several of the structural characteristics of Duchenne Muscular Dystrophy (DMD). Ataluren (PTC124) is proposed to cause read-through of premature stop-codons, and has been introduced as a treatment of genetic disorders. Clinical trials in DMD have shown promise, although with a complex dose dependence. We have established muscle physiology techniques, enabling high resolution of contractile function in zebrafish larvae.

Aim To provide a mechanical analysis of *Sapje* larval muscle and examine effects of Ataluren.

Results Homozygous 5dpf *Sapje* larvae exhibited structural defects with 50 % decrease in active tension. Ataluren (0.1–1 μM, 3–5 dpf) improved contractile function with a maximum of about 60 % improvement of force at 0.5 μM. Controls were not affected by this dose. Higher doses (5, 35 μM) impaired contractile function, an effect also observed in controls, suggesting possible unspecific effects.

Conclusion *Sapje* larvae exhibit an impaired contractile performance and provide a relevant DMD model for studies of functional

improvement. Ataluren significantly improved the muscle functions in the *Sapje* larvae, most likely reflecting effects on dystrophin expression. The bell-shaped dose dependence possibly relates to the observation of beneficial effects in the lower Ataluren concentration interval in the initial clinical studies.

VII.P13.

Viral gene delivery restores muscle function in a mouse model of nemaline myopathy

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The neuromuscular disorder nemaline myopathy is characterized phenotypically by skeletal muscle weakness and histologically by presence of protein aggregates. The disease is caused by mutations in the genes encoding sarcomeric proteins. Here we utilized gene therapy to counteract the skeletal muscle weakness in a mouse model of nemaline myopathy carrying a H40Y amino acid substitution in the skeletal muscle α-actin. We injected recombinant adeno-associated viral vectors, containing the alkali myosin light chain 1 gene (*MYL4*), in the left tibialis anterior limb muscle of 4-week old male mice. The right tibialis anterior limb muscle of these rodents served as control and was injected with saline. 3 weeks after the injections, the animals were euthanized and the muscles excised. The expression of *MYL4* was evaluated as well as the structural and functional consequences of the treatment at cellular and molecular levels. In the left tibialis anterior muscle, *MYL4* gene product was expressed and it induced a tremendous increase in the size and force generated by muscle fibres. This gives strong support for future studies using *MYL4* as a treatment for nemaline myopathy to improve skeletal muscle structure and function.

VII.P14.

Proteomic profile of dystrophic skeletal muscles: new potential biomarkers and drug targets for dystrophinopaties

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Duchenne muscular dystrophy is a childhood myopathy characterized by progressive muscle degeneration and cardiorespiratory failure. In the present study we used the multidimensional protein identification technology (MudPIT) method for shotgun proteomic approach combined with tandem mass tags (TMT) labels to analyze the diaphragm (DIA) of 2-month-old dystrophin deficient *mdx* mice. We examined compensatory mechanisms involved in dystrophic muscle protection against myonecrosis, by comparing the proteomic profiles from *mdx* DIA against the one from *mdx* extraocular muscles (EOM), which do not show myonecrosis. Out of the 857 identified proteins, 42–62 proteins had differential abundance. The calcium-handling proteins sarcalumenin and calsequestrin-1 were increased in control EOM compared with control DIA, suggesting that constitutional properties of EOM are important for their protection against myonecrosis. Proteins involved in oxidative stress response (HSP 75, serpin H1, peroxiredoxin-2), muscle regeneration (galectin-1), inflammation (annexins A1 and A5) and fibrosis (HSP 47) were increased in the *mdx* DIA compared to control DIA suggesting novel mechanisms through which *mdx* affected muscles are able to modulate dystrophy during this early stage of the disease. The present proteomics study of the dystrophic DIA demonstrates an increase in proteins involved in different pathophysiological mechanisms, making them valuable candidates as potential biomarkers and drug targets for dystrophinopaties.

VII.P15.

Tropomyosin mutations that cause gain of function in skeletal muscle diseases

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Mutations in tropomyosin have been found as the cause of skeletal muscle diseases, such as Nemaline myopathy, CFTD and Cap disease. Tropomyosin interacts with actin in a stoichiometry of 1:7 to regulate muscle contraction and has a sevenfold repeated motif in the positions where it interacts with the 7 actin molecules. One conserved interaction is between amino acids K6, K48, R90, K128, R167, K205 and R244 in tropomyosin and Asp25 in actin. Four of these amino acid repeats share the same motif, with two basic amino acids next to each other: K6–K7, K48–K49, R90–R91, R167–K168. Interestingly, mutations in all of these have been reported in skeletal myopathies. It has been shown that β tropomyosin mutation ΔK7 caused increased Ca^{2+}-sensitivity sliding speed and force showing gain of function. We hypothesized that mutations in all four motifs cause a gain of function. We found that ΔK7, ΔK49, K168E and R91G mutations, previously found in skeletal myopathies, causes a gain of function when incorporated into thin filaments, manifested as increased Ca^{2+}-sensitivity determined by the quantitative in vitro motility assay. Similar increases in Ca^{2+}-sensitivity were observed with 100 and 50 % mutant protein, which represents better the physiological condition. In contrast, mutations in nearby amino acids also found in myopathy patients, R167H and R245G, result in a lower Ca^{2+}-sensitivity and loss of function. We propose that these mutations specifically destabilise the tropomyosin- actin complex in the closed (C) state, leading to a gain of function and thus a hypercontractile phenotype in patients.

VII.P16.

Extracellular matrix re-modelling during regeneration in normal and dystrophic skeletal muscle

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The relationship between extracellular matrix (ECM) protein accumulation and skeletal muscle regeneration is important for the development of therapies for diseased and injured muscle. ADAMTS5 is an ECM remodeling enzyme that specifically cleaves ECM proteoglycans, such as verscian, aggrecan, and decorin. Based on reports of elevated ADAMTS5 levels in mdx muscle, which is the mouse model

of Duchenne Muscular Dystrophy, we crossed an Adamts5$^{-/-}$ mouse with the mdx model to generate an Adamts5-/- mdx mouse. It was hypothesised that genetic ablation of Adamts5 would ameliorate the disease pathogenesis in mdx muscle. The mdx pathology is characterised by elevated inflammation and acute muscle necrosis at 3 weeks of age, which leads to an ongoing cycle of muscle degeneration and incomplete regeneration. Initial muscle damage was assessed by in situ functional testing of the tibialis anterior muscle, while the involvement of ADAMTS5 and its substrate targets in the dystrophic inflammatory pathway were also investigated by molecular and immunohistochemical techniques. Before characterising the Adamts5$^{-/-}$ mdx phenotype, it was important to examine the role of ADAMTS5 in wild type skeletal muscle. The absence of ADAMTS5 did not result in a muscle phenotype in sedentary C57/BL6 mice, but the response to voluntary exercise did elucidate some novel metabolic and fibre type results which were associated with differences in fatigue resistance.

II.P17.

Mutation in BAG3 results in severe myofibrillar myopathy and Z-disc disintegration

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BAG3 also known as Bcl-2-associated athanogene 3, Bcl-2-binding protein Bis or docking protein CAIR-1 belongs to BAG family of molecular chaperone regulators. It interacts with Hsp70 and anti-apoptotic protein Bcl-2, and thus is involved in a panoply of cellular processes such as development, apoptosis, autophagy as well as cytoskeleton organization, cell adhesion and motility. *BAG3* is ubiquitously expressed in all tissues with the strong expression in skeletal and cardiac muscle. *bag3* deficiency in mice resulted in fulminant myopathy and early lethality, and mutations within *BAG3* caused progressive limb and axial muscle weakness as well as respiratory insufficiency and neuropathy.

We report Polish 12-year-old girl with heterozygous Pro209Leu (*626C > T*) mutation and clinical symptoms characteristic for *BAG3* mutations described so far. Analysis of quadriceps muscle biopsy revealed disintegration of Z-discs, extensive accumulation of granular debris and larger inclusions within muscle fibers. Indirect immunohistochemistry of proband's muscle revealed profound alterations in BAG3 distribution in comparison with control subject's muscle. The protein localized to long filamentous structures present across the proband's muscle fibers. These structures were also positively immunostained with anti α-actinin antibody indicating their association with disintegrated Z-discs. The data provide further evidence that BAG3 is associated with Z-line maintenance, and mutation within its gene lead to severe myofibrillar myopathy in young age.

VII.P18.

The effect of the Glu41lys mutation in β-skeletal tropomyosin on its position on the thin filament and flexibility during the ATPase cycle

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The nemaline myopathy and cap disease-associated Glu41Lys mutation in β-tropomyosin (TM) has been shown to alter contractile regulation. To study how the Glu41Lys mutation affects the position and flexibility of TM on the thin filament, we labelled recombinant wild type and mutant β-TMs with 5-IAF and F-actin with FITC-phalloidin, and incorporated them into ghost muscle fibres. The orientation and mobility of the probes were studied by polarized fluorimetry at different stages of the ATPase cycle. Multistep alterations in the position and flexibility of β-TM strands and actin monomers in the thin filaments were observed. In the absence of myosin heads the Glu41Lys mutation was found to shift tropomyosin strands towards the outward domain of actin, exposing few myosin-binding sites. The Glu41Lys mutation inhibited myosin-induced shift of TM strands towards the open position, markedly changing the flexural rigidity of both F-actin and TM strands and decreasing the amount of actin monomers capable of forming cross-bridges. This indicates that the number of the cross-bridges that are strongly bound during of the ATPase cycle is reduced. These structural changes in the thin filament are likely to underlie the observed decrease in Ca^{2+}-sensitivity caused by this mutation, the alteration which plausibly initiates the disease. The work was supported by the Russian Fund for Fundamental Research (№ 11-04-00244a), the Programme of Presidium of RAS (theme No. 7) and the Muscular Dystrophy Campaign.

VII.P19.

Distal arthrogryposis-associated Arg91Gly mutation in β-tropomyosin induces the structural changes in the thin filaments during the atpase cycle

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The distal arthrogryposis (DA) is a serious congenital disease characterized by various muscle deformities and contractures of distal limbs such as hands and feet. Mutations in the three genes TPM2, TNNT3, and TNNI2 were identified at the pathogenesis of DA. The aim of the present study has been to investigate the effect of Arg91Gly point mutation in β-tropomyosin (β-Tm) molecule from skeletal muscles on its position and flexibility in the thin filaments. For this purpose the fluorescent probes, FITC-phalloidin and 5-IAF, specifically bound to F-actin and mutant β-Tm, respectively in the ghost muscle fibers (GF) were used. The Arg91Gly mutation in TPM2, encoding skeletal β-tropomyosin associated with DA has been investigated by the method of polarized fluorimetry. During experiments the spatial orientation and mobility of the probes at several simulated stages of ATP hydrolysis cycle were assessed. In the thin filaments the flexural rigidity of F-actin was less than half the rigidity of either of Tms. In the absence of myosin heads the mutation in TM was found to transfer tropomyosin strands towards the inner domain of actin. Incorporation of myosin heads (S1) into the GF causes further displacement of Tm strands towards actin inner domain apparently uncovering more myosin-binding sites. Thus the number of strongly bound S1 molecules increases at all the stages of the ATPase cycle. These structural changes in the thin filaments may partially be explained by the earlier reported increase in Ca^{2+}-sensitivity caused by the point mutation Arg91Gly that leads to myopathy. The work was supported by the Russian Foundation for Basic Research (No. 11-04-00244a), the RAS Presidium Program (theme No. 7) and the Muscular Dystrophy Campaign.

VII.P20.

DMD transcript imbalance regulates dystrophin levels

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Duchenne and Becker muscular dystrophies (DMD and BMD) are caused by respectively out-of-frame and in-frame mutations in the dystrophin encoding *DMD* gene. Molecular therapies targeting either the pre-mRNA, such as antisense oligonucleotides (AONs), or the mRNA, such as read-through of stop codons, are in clinical trials and show promising results. The effect of these approaches will depend on the stability and expression levels of the DMD mRNA in skeletal muscles and heart. We report that the DMD transcript is more abundant in heart compared to skeletal muscles, in mouse and man. We show that the transcript shows a significant 5′-3′ imbalance in wild type mice, which is more pronounced in the *mdx* mouse model carrying a nonsense mutation. Reading frame restoration via antisense-mediated exon skipping did not correct this imbalance. Finally, we demonstrate that transcript balance, but not transcriptional rate is an important determinant for dystrophin protein levels in 22 Becker patients. We suggest that the availability of the entire transcript is a key factor to determine protein abundance and this may influence the outcome of mRNA targeting therapies.

VII.P21.

The cause of developmental myopathy due to the embryonic myosin heavy chain Thr178ile mutation

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Embryonic myosin heavy chain (MyHC), encoded by *MYH3*, is expressed during fetal development and its expression declines rapidly after birth. The essential role of embryonic MyHC for fetal development has been highlighted by the identification of dominant *MYH3* mutations associated with distal arthrogryposis (DA) syndromes. DA is a clinically and genetically heterogeneous group of disorders characterized by flexion of the joints with congenital contractions. It has been hypothesized that the congenital contractions result from reduced fetal movement and severe myopathy during a period of fetal development. Due to lack of muscle biopsy from embryos and suitable animal models, it is not yet established how *MYH3* mutations cause DA.

Here we address the pathogenesis of developmental myopathy caused by heterozygocity for the Thr178Ile *MYH3* mutation, using cultured myotubes from a DA patient as an experimental disease model. Both mutant and wild-type alleles were expressed equally at the mRNA level. An increased expression level of both embryonic and fetal MyHCs in cultured myotubes from the patient compared to healthy controls was observed. Cultured myotubes from patient showed delayed myofibrylogenesis and reduction of cell diameters.

The results suggest that developmental myopathy caused by Thr178Ile *MYH3* mutation is associated with expression of mutant isoform and abnormal muscle development. Consequently, the expression of mutated embryonic MyHC may cause structural and/or functional abnormalities leading to dysfunctions during fetal development.

VII.P22.

Satellite cells in muscles of terminal ALS patients

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Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disorder characterized by loss of motor neurons, progressive paralysis and muscle wasting. The extraocular muscles (EOMs) are less affected than the limb muscles of the same donor in terminal ALS.

Here we investigated whether differences in regenerative capacity may explain the relative sparing of the EOMs.

EOM and limb muscles from 8 ALS donors were cross-sectioned and treated with antibodies against Pax7, NCAM and laminin to quantify satellite cells (SCs) in EOMs, biceps brachii, vastus lateralis and gastrocnemius muscles. Over 30,000 muscle fibres from 28 samples were analysed, mean and range SCs/fibre were: EOM 0.022 (0.015–0.028), biceps 0.053 (0.023–0.093), vastus 0.068 (0.055–0.086), gastrocnemius 0.064 (0.042–0.095), which are similar to previously reported values for old, healthy individuals. An exception was found in two donors with a $SOD1^{D90A}$ mutation and unusually long survival, who had two to threefold higher numbers of SCs in their lower extremity muscles, but not in their EOMs.

Previous data from cell cultures and mouse models suggest that decreased function and/or quantity of satellite cells (SCs) play a role in ALS. Our data show that altered SCs quantity is neither prominent in EOM nor limb muscles in end-stage ALS. The exceptions found prompt further investigations into the possible role of SCs for slower progression in familial ALS with $SOD1^{D90A}$ mutation.

VII.P23.

Slow type fiber phenotype is involved in skeletal muscle dysfunction in an EDMD mouse model

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Emery-Dreifuss muscular dystrophy (EDMD) is characterized by early joint contractures, slowly progressive muscular wasting and weakness, and by adult age, development of cardiac conduction defects, arrhythmias, left ventricular dysfunction and dilation with heart failure. Autosomal dominant EDMD is caused by mutations in *LMNA* gene encoding lamins A/C, two components of the nuclear envelope. We developed a knock-in mouse model, $Lmna^{H222P}$ reproducing a missense *LMNA* mutation (p.H222P) identified in a family with typical EDMD. These mice develop phenotype mimicking the human EDMD disease.

Skeletal muscles from young (4 months) male $Lmna^{H222P/H222P}$ mice showed no alteration of tension and of kinetics of contraction and relaxation. However, in older $Lmna^{H222P/H222P}$ mice (6 months), the slow-twitch *soleus* muscle presented a 50 % reduction of tension compared to WT *soleus* muscle, whereas the fast-twitch *extensor*

digitorum longus muscle contracted normally. RT-qPCR from *soleus* of 6-month old $Lmna^{H222P/H222P}$ mice indentified down regulation of 3 genes (*Myh7*, *Myh2* and *Tnnc1*) involved in the slow type fibres phenotype compared with WT. We also identified from the same mice up regulation of *Nmrk2* and down regulation of *Nampt*, two major actors of the nicotinamide adenine dinucleotide (NAD) metabolism. In parallel open field experiments failed to reveal any modification of the motor activity of those mice.
In conclusion the decrease of tension in the *soleus* of the 6-months old $Lmna^{H222P/H222P}$ mice is related to defective slow type fibres, which is not a consequence of decreased motor activity and seems mediated through new signalling pathway involving the NAD metabolism.

VII.P24.

Novel ANO5 antibodies reveal protein defects in anoctaminopathy patients

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Mutations in *ANO5* cause recessive muscular dystrophies with proximal and/or distal phenotypes including LGMD2L and Miyoshi-like muscular dystrophy (MMD3). Anoctamin 5 (ANO5) is a putative multipass membrane protein and Ca^{2+}-activated Cl^--channel, highly expressed in skeletal and cardiac muscle, reported to localize in the sarcoplasmic reticulum membranes. To date, detecting ANO5 protein in human biopsies has proved difficult with the available commercial antibodies.
We performed Western blot experiments on muscle protein extracts from 9 Finnish patients with 5 different compound heterozygous or homozygous mutations in the *ANO5* gene. Two rabbit polyclonal antibodies raised against N- and C-terminal peptides of mouse ANO5 protein were used for WB hybridization.
Here we report that the two rabbit polyclonal antibodies recognize two proteins approximately located at 160 and 50 kD, which show variable reduction in anoctaminopathy patients, being more severe in biopsies with truncating mutations. The predicted molecular weight of the ANO5 polypeptide is 107 kD. The observed discrepancy in MWs may be caused by covalent modifications of the full-length ANO5, increasing its size (160 kD band), and the 50 kD band is probably a proteolytic fragment.
The western blotting method described here is able to produce robust results on ANO5 protein expression, and it may be of diagnostic use.

VII.P25.

Force–sarcomere length relations in patients with thin filament myopathy caused by mutations in *NEB*, *ACTA1*, and *TPM3*

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Background Mutations in the nebulin gene (*NEB*), skeletal muscle alpha-actin1 gene (*ACTA1*), and alpha-tropomyosin 3 gene (*TPM3*) lead to thin filament myopathies, such as nemaline myopathy (NM), congenital fiber type disproportion (CFTD) and cap disease (CAP). A hallmark feature of these myopathies is muscle weakness. Here, we aimed to elucidate the effect of *NEB*, *ACTA1* and *TPM3* mutations on thin filament length by determining the sarcomere length-dependence of force.
Methods Quadriceps biopsies from NM, CFTD, and CAP patients ($n = 16$) with mutations in either the NEB, ACTA1, or TPM3 were compared to biopsies from controls ($n = 3$). Using permeabilized muscle fibers, maximal active tension was determined at incremental sarcomere lengths (range 2.0–3.5 μm) to obtain the force-sarcomere length relationship.
Results The maximal active tension and the force–sarcomere length relationship data are summarized in the table below (a *leftward shift* of the force–sarcomere length relation indicates shorter thin filaments). Data are presented as mean ± SD.

Maximal active tension (mN/mm^2)	CTRL	*ACTA1* mild form (based on age of onset)	ACTA severe form (based on age onset)	*NEB* mild form (based on age onset)	*NEB* severe form (based on age onset)	*TPM3* mild form (based on age onset)	*TPM3* severe form (based on age onset)
	164 ± 17	139 ± 27	54 ± 13[a]	76 ± 5[a]	18 ± 5[a]	156 ± 13	95 ± 14[a]
Shape of force–sarcomere length relation		Unaffected	Modest leftward shift	Modest leftward shift	Marked leftward shift	Unaffected	Unaffected

[a]Significantly lower compared to CTRL

Note that, in contrast to patients with *ACTA1* and *TPM3* mutations, fiber preparations from both *mildly* and *severely* affected *NEB* patients have a disturbed force-sarcomere length relation.
Conclusion Our data suggest that mutations in *NEB* result in the most pronounced changes in thin filament length. Insights in the mechanisms underlying weakness in patients with thin filament mutations are necessary to improve specific treatment strategies.

POSTER SESSION VIII Smooth muscle

VIII.P1.

Elusive role of synaptopodin 2 in smooth muscle cell phenotype switch

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The actin binding protein synaptopodin 2 (SYNPO2), is a dual compartment protein that shuttles between the nucleus and the cytosol. Its migration from the nucleus into the cytoplasm is supposed to be linked to the differentiation of striated muscle cells (Weins et al. 2001). Most investigations on SYNPO2 were made in striated muscle cells while little is known about its physiological function in smooth muscle cells.

Smooth muscle cells feature the ability to switch their appearance from a contractile to a synthetic phenotype and vice versa, depending on factors like confluence, support matrix, and growth factors. Purpose of our research is to find out (a) the consequences of this switch on the subcellular localization of SYNPO2 and (b) whether SYNPO2 plays an active role in this process.
We cultured primary smooth muscle cells from rat aorta on different matrices. Classification into the two smooth muscle phenotype classes was based on the morphological appearance and on the expression of the markers α-actin, smooth muscle myosin heavy chain, calponin, h-caldesmon, SM22α and smoothelin. Localization of SYNPO2 was determined by immunofluorescence microscopy and subcellular fractionation followed by gel electrophoresis and western blots.
We were able to drive smooth muscle cells into either a cobble stone (synthetic) or a spindle form appearance by using collagen and laminin, respectively. However, subcellular localization of SYNPO2 did not accompany a change in shape. Thus, the precise involvement of SYNPO2 in smooth muscle cell phenotype change remains obscure.

VIII.P2.

Gene therapy by recombinant plasmid with human nerve growth factor gene stimulates angiogenesis and restoration of blood flow in ischemic murine hind limb

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Obliterative atherosclerosis of lower limb arteries leads to onset of severe ischemia and increases overall morbidity and mortality. Surgical methods have limited availability and thus there is need for development of alternative reperfusion methods. One of them is therapeutic angiogenesis by growth factor genes, which stimulate formation of new vessels in ischemic tissue.
Our study intended to evaluate possibility of angiogenesis stimulation via local delivery of plasmid construct with human nerve growth factor (hNGF) gene. We used a murine limb ischemia model to assess the plasmid angiogenic potential in vivo. Plasmid DNA was diluted in saline and injected to ischemic m. tibialis anterior. Blood flow restoration was measured by laser Doppler every 7 days after the surgery. Throughout the experiment we assessed animal's feet necrosis and limb. After animals were sacrificedmuscle samples were frozen for histological analysis. Tissue sections were stained by antibodies against endothelium marker CD31 to assess vascular density.
Macro-analysis of limbs status showed that animals from NGF treatment group had fewer edema signs and inflammatory changes in ischemic and surgical wound area. Blood perfusion by day 7 was higher in NGF group compared to control ($p = 0.01$) and by day 14 NGF group had perfusion 2.8 fold higher than in control animals (NGF 44.62 ± 7.68; control 16.74 ± 5.85; $p = 0.005$). Vascular density in tissue samples by at day 14 in NGF-treated animals was 2.5 fold higher than in control ($p < 0.05$).
Our data suggests that plasmid delivery of hNGF is an effective therapeutic tool for angiogenesis stimulation.

VIII.P3.

Correlation between contractile kinetics and energy metabolism in smooth muscle tissues

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Background Smooth muscle is slow and economical in comparison to striated muscle, but exhibits a large span in contractile properties, from the fast phasic types in visceral organs to the slow and tonic types in the large arteries. These contractile properties are associated with different myosin isoforms and proteins in the activation/deactivation pathways. Although the mechanical output and cellular regulation have been extensively investigated in smooth muscle, pathways in the energy supply to the contractile system have received less attention.
Aim We have initiated a profiling study of mRNA for enzymes in the smooth muscle energy metabolism to examine if the contractile kinetics are coupled to specific metabolic properties.
Results Smooth muscle from ileum, urinary bladder, trachea, saphenous artery and aorta from mouse were examined with qPCR and compared with heart and skeletal muscles. Smooth muscle contractile type was determined from the expression of the alternatively spliced heavy and light chain myosin isoforms. The slow smooth muscles (arterial) were characterized by high expression of enzymes in the lipid synthesis and uptake (Acetyl-CoA carboxylase, ACC; Fatty Acid Synthase, FAS; LipoProtein Lipase, LPL; Hormone Sensitive Lipase, HSL), in the glucose uptake (Hexokinase, HK) and in the glucose synthesis (PhosphoEnolPyruvic CarboxyKinase, PEPCK).
Conclusion We show that the large span in contractile properties between smooth muscle is accompanied by significant variation in energy metabolic pathways, where the comparatively slow arterial muscles seem to be adapted for higher rates of glucose and lipid synthesis and uptake.

Tuesday September 24 2013

POSTER SESSION IX Mutations in inherited cardiomyopathies

IX.P1

Unraveling the role of junctophilin-2 for stabilizing the cardiac dyadic cleft and the effects of single point mutations identified in patients with hypertrophic cardiomyopathy

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Excitation contraction coupling in cardiac muscle requires a precise spatial organization of the L-type voltage-gated calcium channel (LTCC), on the t-tubule (t-t) membrane, and the ryanodine receptor (RyR2) localized to the junctional sarcoplasmic reticulum (jSR). In the heart junctophilin-2 (JP2) is thought to act as a scaffold, to bridge and stabilize the gap between the t-t and jSR membranes forming the dyadic cleft. Genetic defects in JP2 (S101R, S165F, Y141H, G505S) have been associated with hypertrophic cardiomyopathy (HCM), the most common cause of sudden cardiac death in young people. The mechanisms by which these mutations lead to disease are still not understood. In order to investigate how the introduction of single point mutations may influence the function of JP2 we have expressed and purified wild-type and mutant JP2 proteins from *E. coli*. Using a combination of fluorescence spectroscopy, circular dichroism, thermal and chemical denaturation protocols we have examined the effects of each mutation upon the physiochemical properties of JP2. In summary, experiments revealed that the mutations had no overall effect on protein stability. However, the S101R and Y141H mutants exhibited changes to the secondary structure profile with a reduction to the overall helical content and increase in random coil content. These results indicate that the JP2 mutations do not appear to effect

the stability of the protein but lead to a modification of the structure which may alter the lipid binding properties and/or disrupt potential protein interactions between JP2 and dyadic cleft proteins involved in calcium homeostasis.

IX.P2.

HCN and SCN5a channel mutations: implications for impaired atrioventricular nodal conduction in heterogeneous computer models of the rabbit and mouse heart

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The atrio-ventricular node (AVN) is a specialised region of the cardiac conduction system, allowing electrical excitation to propagate from the atria to the ventricles. AVN block occurs when the AVN prevents electrical stimuli reaching the ventricles, ultimately leading to a decrease in cardiac output and possible cardiac death. HCN and SCN5A genetic mutations have previously been identified in patients with AVN block, but the mechanisms underlying impaired AVN conduction remain incompletely understood. The aim of this study was to assess the functional impacts of HCN knockout and SCN5A gene mutations (T220I, P1298L, delF1617 and E161K) on AVN block. We implement a multi species approach for such investigation: HCN knockout and SCN5A gene mutations are incorporated into recently published rabbit and newly developed mouse AVN models. These models, along with models for the sino-atrial node, atria, Purkinje fibre and ventricular tissue, are incorporated into 1D and 2D whole heart models. In rabbit, the models are also mapped onto an anatomically accurate 3D reconstruction of the AVN and surrounding tissue. Both HCN knockout and SCN5A mutations had a significant effect on cardiac pacing (as illustrated by a remarkable reduction in cardiac pacing rate) and excitability. They also caused a slowing of conduction velocity in the conduction system of the heart, particularly in the AVN region, resulting in enhanced susceptibility to AVN block. The underlying ionic and anatomical mechanisms of conduction block are highlighted by the models, leading to indication of the potential targets for drug therapy of AVN block.

IX.P3.

Pro-arrhythmic effects of KCNA5 mutations: insights from the 3D virtual human atria

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Genetic defects are thought to be major factors underlying 'lone' atrial fibrillation (AF) in patients with normal cardiac structures. A recent study has identified 6 novel mutations in the KCNA5 channel, encoding for the atrial specific potassium channel, I_{Kur}. The mutations identified include both gain- (E48G, A305T, D322H) and loss-of-function (Y155C, D469E, P488S) of the channels. I_{Kur} is of potential interest as a target for drug therapy treatment of AF, and hence it is important to elucidate the potentially pro-arrhythmic effects of the identified mutations. Computational modelling provides a useful framework to do so. In this study, we incorporated a new wild type formulation of I_{Kur} into a previous model for the human atrial action potential (AP), along with models for all of the identified mutations. The previously developed 3D atrial model includes multiple protocols for the initiation of AF and hence allows a detailed investigation of the effect of the mutations on re-entrant behaviour during AF. It is demonstrated that the gain-of-function mutations shorten the effective refractory period (ERP) of the AP and promote stable re-entrant activity, whereas the loss-of-function mutations prolong the ERP while also increasing atrial tissue regional AP heterogeneity. The increased regional electrical heterogeneity promotes the breakdown of excitation waves that degenerated into multiple re-entrant wavelets with characteristics of AF. In addition, the loss-of-function mutations produced early-after-depolarisations in the presence of isoprenaline, which can lead to ectopic activity associated with AF. This study identifies the pro-arrhythmic effects of both loss- and gain-of function mutations in KCNA5.

IX.P4.

Stress-induced troponin rise in patients with hypertrophic cardiomyopathy: associations with sarcomere mutation and MRI characteristics

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Inadequate length-dependent activation with sarcomeric dysfunction and microvascular dysfunction are just two different proposed mechanisms of disease in hypertrophic cardiomyopathy (HCM). Potentially, ischemia may finally occur with both as the underlying mechanism, especially during exercise, with the same net result: myocardial injury. We investigated whether exercise can elicit cardiac troponin release from the hypertrophied heart, and if so which factors are associated with the stress-induced release.
In 94 genotyped HCM patients a bicycle exercise stress test was performed. Before and 6 h after the exercise test troponin T was measured using a highly-sensitive troponin assay. A troponin rise was defined as a >20 % rise in concentration. Prior to the exercise test cardiac MRI was performed to assess the presence of myocardial edema and fibrosis. Troponin rise occurred in 32 patients. There were no differences between sarcomere mutation negative and positive patients with regard to the incidence of troponin rise. Also, the duration of exercise and LV mass did not differ between patients with or without a rise in troponin concentration. Patients with a troponin rise were more likely to have imaging evidence of edema and fibrosis. We conclude that a troponin rise was present in a third of our patients. Interestingly, stress-induced troponin rise was associated with edema on MRI and the presence of fibrosis, whereas sarcomere mutation status and LV mass, both potential causes of recurrent episodes of ischemia, were not.

IX.P5.

Isolated dilated cardiomyopathy with dyspnea in the patient with dystrophin gene mutation

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Neuromuscular disease is the second common cause of dilated cardiomyopathy (DCM). Most of the cause of DCM in neuromuscular disease is Duchenne/Becker muscular dystrophy so called dystrophinopathy. But these patients commonly presented muscle weakness than cardiomyopathy. We report a Becker muscular dystrophy patient who had a dilated cardiomyopathy without skeletal muscle weakness.
A 26-year old man visited with dyspnea. Since he was young, running was slower than others. At the age of middle school, he felt intermittent mild dyspnea when climbing upstairs, but he didn't feel any difficulty of usual life. At the age of 26, he felt exertional dyspnea. He visited our cardiologic department and revealed pulmonary effusion caused by heart failure.
At the time of physical examination, bilateral calf hypertrophies were seen but there was no Gower's sign. Cranial functions were normal and facial weakness is not seen. The MRC motor grade checked grade V in bilateral proximal and distal muscles. Heel gait and toe gait were normal. He felt minimal difficulty when squatting to standing 10 times, but he didn't holding his knee when squatting to standing. He had a family history of neuromuscular disease, two male of matrilineal cousin had muscle weakness. Serum creatinine kinase (4,020 U/L) and CK-MB (43.21 U/L) were elevated. Electrocardiogram showed sinus bradycardia (49 bpm) and right bundle branch block. Transthorcic echocardiography revealed enlarged left atrium (LA) and ventricle (LV), global LV systolic dysfunction (EF = 24 %), and severe global hypokinesia of LV. These findings were compatible with dilated cardiomyopathy. The dystrophin gene showed duplication of exons 14–29 by MLPA (multiple ligation probe amplification) method.
There have been several studies to distinguish phenotypes in dystrophinopathy. And has been known degree of skeletal muscle involvement and cardiomyopathy is not proportional. Our patient has a cardiomyopathy witout significant skeletal muscle weakness until adult age. This is a meaningful case to phenotype evaluation and to understand dystrophinopathy.

IX.P6.

How can diverging functional effects of myosin mutations in FHC induce a similar disease phenotype?

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The initial triggers of Familial Hypertrophic Cardiomyopathy (FHC) are still largely unknown. Currently it is thought that FHC-myofilament mutations cause higher calcium-sensitivity, force and ATPase activity. We asked whether this holds also true for ß-myosin missense mutations and performed functional studies on slow skeletal muscle fibers that also express ß-myosin and which we isolated from FHC-patient's muscle biopsies.
Our experiments revealed that among 7 different β-myosin heavy chain mutations, only 3 showed enhanced F_{max} (R723G, R719 W and R453C) and one out of five mutations showed higher calcium-sensitivity (G741R). Reduced force generation and calcium-sensitivity was also found, while for mutations V606 M and G584R no functional alterations were detected.
Quite surprisingly, we observed a marked variability in pCa_{50} among individual fibers carrying the same mutation, ranging from almost normal response to highly significant differences. Such large functional variability was not observed in control fibers. To clarify the reason for this we quantified the relative abundance of mutated vs. wildtype ß-myosin-mRNA in single fibers. We found variable expression of mutated myosin from fiber to fiber that may be responsible for the observed unequal force generation.
We conclude that there is not a specific primary functional effect common to all FHC-mutations. Instead, we hypothesize that unequal expression of mutated and wildtype myosin also occurs in individual cardiomyocytes of the patients, leading to imbalanced force generation of cardiomyocytes in systole. We propose that allelic imbalance is a trigger common to all myosin mutations, setting off altered cell signaling and morphology like myocyte and myofibrillar disarray.

IX.P7.

Ca^{2+}-homeostasis in ventricular cardiomyocytes and atria from transgenic mice carrying the FHC-linked deletion mutation cTNI-k184

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The FHC related cTnI-K184 transgenic (TG) mouse exhibits signs of dystolic dysfunction in vivo and at the myofibrillar level. Here, we investigated whether Ca^{2+}-homeostasis is altered in ventricular cardiomyocytes (VCM) and atria isolated from these mice. In electrically stimulated (0.5 Hz) VCM loaded with fura2-AM, basal $[Ca^{2+}]$, the amplitude and the rate of the decay of the Ca^{2+}-transient were similar in TG and WT. Increasing stimulation frequency to 2 Hz increased basal $[Ca^{2+}]$ in TG by $\sim$25 % ($p < 0.05$). Left atria mounted in a myograph were electrically stimulated with 4 Hz. Compared to WT, twitch amplitude (TA) was lower in TG (3.1WT versus 2.8TG, $p < 0.5$), while the time constant τ of relaxation was significantly larger (21 s TG versus 13 s WT, $p < 0.001$). Interrupting stimulation for 5 to 120 s, which has been suggested to change the driving force for NCX, resulted in postrest potentiated contractions (PPC). PPC declined exponentially during subsequent twitches to TAs similar to control twitches before rest (CTA) in TG for all pauses while in WT, PPC declined to lower values than CTA for pauses >90 s. The calculated re-circulating Ca^{2+}-fraction for pauses >90 s was $\sim$74 % in WT and $\sim$81 % in TG ($p < 0.05$). Whether this can be ascribed to a lower NCX activity in TG relative to WT remains to be determined. In conclusion, the cTnI-K184 mutation induces subtle changes in calcium homeostasis likely contributing to diastolic dysfunction at the cellular level and in vivo in our FHC mouse model.

IX.P8.

Most prevalent hypertrophic cardiomyopathy associated *MYBPC3*$^{\Delta C10}$ mutation causes contractile dysfunction in vitro

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Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease. HCM is caused by mutations in genes encoding for sarcomeric proteins and 40 % of identified mutations are in cardiac

myosin binding protein-C (cMyBP-C) gene. Recently, an HCM-associated 25 base pair deletion in the cMyBP-C gene, that is estimated to occur in 55 million people, was identified resulting in replacement of 62 amino acids with 55 novel amino acids in the C-terminal C10 domain (cMyBP-$C^{\Delta C10}$). However, the mechanism by which this mutation causes HCM is unknown. We aim to elucidate the pathogenic mechanism of cMyBP-$C^{\Delta C10}$ expression on sarcomere function.
In this study, homozygous cMyBP-$C^{\Delta C10}$ mutation carriers were identified that presented with cardiac arrhythmia and HCM. Infection of isolated adult rat cardiomyocytes with cMyBP-$C^{\Delta C10}$ adenovirus resulted in incorporation of mutant protein into the sarcomere, with partial replacement of endogenous cMyBP-C. Unloaded cell shortening revealed impaired fractional shortening after cMyBP-$C^{\Delta C10}$ expression. To assess the structural consequences of cMyBP-$C^{\Delta C10}$, circular dichroic analysis was used. Cardiac MyBP-$C^{\Delta C10}$ protein exhibited substantial differences in secondary and tertiary structure compared to wild-type cMyBP-C. Molecular modeling of the C10 domain reveals potential structural perturbation in cMyBP-$C^{\Delta C10}$, which may disrupt the predicted light meromyosin (LMM) binding region.
In conclusion, the HCM associated cMyBP-$C^{\Delta C10}$ protein incorporates into the sarcomere and causes a reduction in contractility. cMyBP-$C^{\Delta C10}$ leads to structural differences in the C10 domain, which are predicted to impact on binding to LMM, which is vital for proper function of cMyBP-C to regulate cardiac muscle contractility.

IX.P9.

Mutations in giant sarcomeric proteins discovered by whole exome sequencing of heart tissue from patients with familial DCM

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Dilated cardiomyopathy (DCM) can be caused by mutations in a large number of structural proteins, but the effects of these mutations and the underlying molecular mechanisms remain not well defined.
Here we studied genomic DNA obtained from 12 muscle samples from explanted hearts of patients diagnosed with familial DCM and applied whole exome sequencing. We investigated variants in 58 sarcomomeric and cytoskeletal genes previously linked with DCM.
Variants found in the general population and in multiple samples were excluded and the remainder were filtered for potential disease-causing mutations using SIFT and PPH programs.
Initial analysis revealed 4 chain termination mutations in titin (TTN), 3 mutations, including 1 chain termination mutation in obscurin (OBSCN) and 1 mutation each in myomesin (MYOM1), beta myosin heavy chain (MYH7), troponin C (TNNC1) and desmoplakin (DSP). One sample had both DSP and OBSCN mutations and in 3 samples no mutation was found.
The TNNC1 mutation had been previously identified in this tissue sample, thus confirming the accuracy of the sequencing and analysis. The MYH7 mutation, E1426K, has previously been identified as a DCM-causing mutation in a 3 generation family. The frequency of TTN mutations corresponds to that found by Hermann et al. (NEJM 2012) in 312 DCM samples.
Investigation of titin by gel electrophoresis of the tissue samples indicated substantial haploinsufficiency in two of the four putative titin truncation mutations and a decreased N2BA/N2B ratio in all the DCM samples independent of the protein mutated.

IX.P10.

The effects of the Ca^{2+}-sensitiser EMD57033 on cardiac muscle contractility parallel HCM-causing mutations

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Hypertrophic Cardiomyopathy (HCM) mutations in sarcomeric proteins are proposed to increase myofibrillar Ca^{2+}-sensitivity and some also uncouple Ca^{2+}-sensitivity changes from changes in troponin Iphosphorylation. EMD 57033 is a Ca^{2+}-sensitiser and is reported to increase myofibrillar Ca^{2+}-sensitivity. We investigated how the effects of EMD 57033 compared with HCM mutations.
In the in vitro motility assay (IVMA), EMD 57033 increased Ca^{2+}-sensitivity of human non-failing cardiac thin filaments, the effect was maximal at 30 μM with an EC_{50} of 15–20 μM. The increase in Ca^{2+}-sensitivity due to EMD 57033 was threefold for fraction motile and fivefold for motile velocity. In the presence of EMD 57033, Ca^{2+} sensitivity was the same at both high and low levels of troponin I phosphorylation, whilst in the control, dephosphorylation caused a twofold increase in Ca^{2+}-sensitivity. In comparison, the HCM mutation K280 N in TNNT2 increased Ca^{2+}-sensitivity twofold (not as large an increase as with EMD57033). In addition, the Ca^{2+}-sensitivity was independent of the level of phosphorylation of troponin I phosphorylation. In electrically stimulated papillary muscle, EMD 57033 increased the peak force of contraction 1.8-fold in non-transgenic mice. The HCM mutation E99 K in ACTC increased the peak force 3.2-fold compared to non-transgenic. EMD57033 had no effect on the E99 K peak force and therefore their Ca^{2+}-sensitising effects are not additive.
The Ca^{2+}-sensitiser EMD 57033 increases Ca^{2+}-sensitivity and it also uncouples Ca^{2+}-sensitivity from changes in troponin I phosphorylation. The effects on regulation of contractility parallel the effects seen with the HCM mutations TNNT2 K280N and ACTC E99K, also due to Ca^{2+}-sensitivity increases.

IX.P11.

Effects of β-adrenergic signalling on myofibrillar Ca^{2+}-sensitivity and relaxation kinetics in a mouse model of FHC carrying the mutation $cTnI^{\Delta K184}$

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PKA-mediated phosphorylation of contractile proteins upon β-adrenergic stimulation results in a decrease in myofilament Ca^{2+}-sensitivity and an increase in the rate of relaxation. In this study, we addressed the question whether the effect of ß-adrenergic signalling is blunted in skinned fibers and myofibrils isolated from transgenic mice carrying the FHC-linked $cTnI^{\Delta K184}$ mutation.
Skinned fibers from transgenic (TG) mice exhibited an increased Ca^{2+}-sensitivity compared to wild–type (WT) (pCa_{50}: WT 5.52, TG 5.62). Myofibrillar passive force was enhanced (F_{pass}: WT 3.3 ± 0.4 nN/μm^2, TG 7.1 ± 0.8 nN/μm^2; 15 % of slack length) and relaxation rate was slowed down in TG animals (τ_{rel}: WT 16 ± 2 s^{-1}, TG 10 ± 1 s^{-1}). As PKA had no effect on Ca^{2+}-sensitivity indicating functionally saturated phosphorylation levels of PKA-sites, mice were treated with the ß-adrenoceptor blocker propranolol before sacrifice. Phosphorylation of myofibrillar proteins was similar in untreated WT and TG animals. Propranolol treatment decreased cTnI phosphorylation by ~50 % in both groups. In propranolol-treated WT mice, Ca^{2+}-sensitivity was decreased to pCa_{50} 5.47 and relaxation was slowed down significantly (τ_{rel} 13 ± 2 s^{-1}) but F_{pass} was not altered. In propranolol-treated TG mice, Ca^{2+}-sensitivity was increased (pCa_{50} 5.71), F_{pass} was reduced by ~35 %, and relaxation kinetic

was not affected. In both, WT and TG mice treated with propranolol, PKA decreased Ca^{2+}-sensitivity by 0.2 pCa units. In conclusion, treatment with propranolol affects mechanical parameters differently in WT and TG, but the Ca^{2+}-desensitizing effect of PKA is not blunted by the mutation.

IX.P12.

High passive cardiomyocyte stiffness in a mouse model of hypertrophic cardiomyopathy

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Hypertrophic cardiomyopathy (HCM) is the most common genetic cardiac disorder. Mutations in the gene (*MYBPC3*) encoding cardiac myosin binding protein C (cMyBPC) are a frequent cause of HCM. Diastolic dysfunction is evident in pre-hypertrophic mutation carriers and patients with manifest HCM. We hypothesized that high passive stiffness in addition to an increased myofilament Ca^{2+}-sensitivity of the sarcomeres may underlie impaired diastolic function in HCM.
To test this hypothesis, isometric force was measured in mechanically isolated left ventricle membrane-permeabilized cardiomyocytes from male heterozygous (Het) and homozygous (KI) *Mybpc3*-targeted knock-in mice, carrying a *Mybpc3* point mutation (G > A transition on the last nucleotide of exon 6) associated with HCM in humans (n = 5 per group). Force measurements were repeated after pre-incubating cardiomyocytes with protein kinase A (PKA), the downstream kinase of the β-adrenergic receptors.
Passive force (F-pas) was significantly higher in KI than in Het and wild-type mice (WT). In addition, myofilament Ca^{2+}-sensitivity was higher in both KI and Het compared to WT, evident from a lower EC_{50} (respectively, 2.5 ± 0.1, 2.7 ± 0.1 and 2.9 ± 0.1 μmol/L). PKA treatment reduced Fpas in KI to values observed in WT (Fig. A), while EC_{50} was only partially normalized in KI and Het. In line with a high myofilament Ca^{2+}-sensitivity, troponin I phosphorylation was lower in KI and Het compared to WT. Analysis of cardiac titin isoform composition revealed no significant changes in KI and HET compared to WT.
In conclusion, our data show that diastolic dysfunction may be caused by increased passive tension and Ca^{2+}-sensitivity of the sarcomeres.

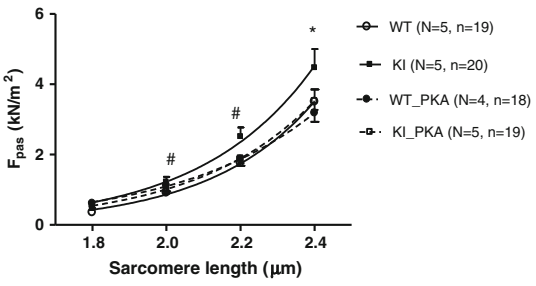

Fig. A Increased passive tension in KI mice, which normalized after PKA incubation. *N* mice, *n* cardiomyocytes. *p<0.05 vs. WT; #p<0.05 vs. corresponding sarcomere length

The defects in sarcomere function are in part due to disturbances in β-adrenergic receptor signaling in HCM with mutant cMyBP-C.

IX.P13.

Altered passive tension in HCM with MYBPC3 mutations

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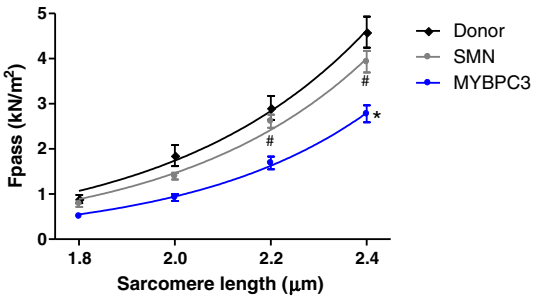
Background Familial hypertrophic cardiomyopathy (HCM) is a heterogeneous cardiac disorder with a prevalence of 1:500. HCM is characterized by asymmetrical thickening of particularly the interventricular septum (IVS) of the left ventricle (LV) without underlying cardiac or systemic disorders. In ~65 % of all HCM patients a mutation is found in genes that encode for the sarcomeric proteins. Mutations in the *MYBPC3* gene encoding cardiac myosin-binding protein C (cMyMP-C) represent >40 % of all genotyped HCM cases. HCM patients with a *MYBPC3* mutation show normal systolic function and impaired relaxation (van Dijk et al. Circulation heartfailure, 2012). Moreover, studies in individuals that carry a *MYBPC3* sarcomere mutation show that diastolic dysfunction is present even before onset of hypertrophy. We hypothesize that increased passive stiffness of sarcomeres underlies diastolic dysfunction in patients with manifest HCM.
Methods In-vivo passive tension measurements were done in cardiomyocytes from 13 patients carrying a MYBPC3 mutation (MYBPC3: 37 cells), 4 sarcomere mutation-negative patients (SMN: 12 cells), and 4 donors (13 cells). Cardiac tissue was obtained during myectomy surgery to relieve LV outflow obstruction. Free wall LV donor tissue was obtained from non-failing donor hearts. Cardiomyocytes were mechanically isolated and Triton-permeabilized.
Results Data are presented as mean ± SEM. Passive tension in MYBPC3 mutants was significantly lower than donors ($p < 0.001$) and SMN ($p < 0.05$; $^{\#}p < 0.01$).
Conclusion In contrast to our hypothesis, passive stiffness was significantly lower in HCM patients with manifest HCM harboring a *MYBPC3* mutation. The lower passive force in MYBPC3 compared to SMN patients suggests that the low passive force is a consequence of the sarcomere mutation.



IX.P14.

Role of fragile X proteins in the heart

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RNA regulation provides a critical mechanism for controlling gene expression in the heart during normal development and disease. The RNA-binding Fragile X (FraX) protein, FXR1, has been linked to proper muscle structure and cardiac hypertrophy in mouse and zebrafish. To decipher the direct role and mechanisms of FraX protein in the heart in vivo, we turned to the genetic capabilities of the *Drosophila* model, which expresses only one, functionally conserved FraX protein (dFmr1). To gain further insights into the role of dFMR1, we sought to identify a functional heart defect in *Drosophila* using video microscopy and a structural defect using immunofluorescence confocal microscopy. Using these approaches, we discovered that loss of dFMR1 causes a statistically significant decrease in heart rate and perturbs sarcomere structure. Expression of human FXR1 partially rescues the functional heart defect loss of function mutants, supporting functional conservation between species. Additionally, using Western blot analysis, we have discovered that loss of dFmr1 alters protein levels of known FXR1 targets: the costameric protein talin and the intercalated disc protein ZO-1. Taken together, these data suggest that dFmr1 has an important role in proper heart function, possibly by the translational regulation of its mRNA targets that encode integral cytoskeleton and cytoskeleton-associated proteins.

IX.P15.

Localisation of AMPK γ subunits in cardiac and skeletal muscles

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The trimeric protein AMP-activated protein kinase (AMPK) is an important sensor of energetic status and cellular stress, and mutations in genes encoding two of the regulatory γ subunits cause inherited disorders of either cardiac or skeletal muscle. AMPKγ2 mutations cause hypetrophic cardiomyopathy with glycogen deposition and conduction abnormalities; mutations in AMPKγ3 result in increased skeletal muscle glycogen. In order to gain further insight into the roles of the different γ subunits in muscle and into possible disease mechanisms, we localised the γ2 and γ3 subunits, along with the more abundant γ1 subunit, by immunofluorescence in cardiomyocytes and skeletal muscle fibres. The predominant cardiac γ2 variant, γ2-3B, gave a striated pattern in cardiomyocytes, aligning with the Z-line but with punctate staining resembling T-tubule and sarcoplasmic reticulum structures. In skeletal muscle fibres AMPKγ3 localises to the I band, presenting a uniform staining that flanks the Z-line. This suggests that AMPK containing γ2-3B or γ3 may be associated with the T-tubules or sarcoplasmic reticulum and that these trimers may have similar functions. The minority γ2 variant, γ2-long, was also detected in the I band with additional staining in the M-line; there is a strong indication that γ2-short (a further γ2 variant) is present in the Z-line where γ1 also localizes. Compartmentalisation of AMPK complexes is most likely dependent on the regulatory γ subunit and this differential localisation may direct substrate selection and specify particular functional roles.

IX.P16.

Altered passive stiffness in human FHC with missense or truncation MYBPC3 mutations

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Background Familial Hypertrophic Cardiomyopathy (FHC) is a cardiac disease that manifests due to mutations in genes encoding sarcomeric proteins. The disease is characterized by asymmetrical thickening of the left ventricle (LV) and interventricular septum (IVS) and diastolic dysfunction. More than 40 % of FHC patients carry a mutation in the MYBPC3 gene encoding cardiac myosin-binding protein C (cMyBP-C). We aim to understand how different mutations in MYBPC3 can cause altered passive stiffness in FHC patients.
Method Cardiac samples were obtained from FHC patients with LV obstruction and from non-failing donors. Passive force measurements were performed in membrane-permeabilized cardiomyocytes. Protein homogenates were run on 1 % Agarose gels and stained with ProQ-Diamond and SYPRO Ruby to determine total titin phosphorylation and isoform composition.
Results Passive tension was found to be significantly lower in MYBPC3 mutants with missense and truncation mutations compared to donors (2.16 ± 0.27, 1.46 ± 0.15, 2.90 ± 0.26, respectively). The passive tension of patients with truncation mutations was significantly lower compared to those with missense mutations. MYBPC3 patients were found to have slightly higher N2BA:N2B expression compared to donors, indicating a higher expression of the more compliant titin isoform. Truncation mutants showed a significantly higher ratio of phosphorylated N2BA to phosphorylated N2B compared to missense mutants and donors.
Conclusion Differential passive stiffness was found between patients with missense and truncation mutations in MYBPC3 compared to donors. These data indicate that the type of mutation in the same sarcomeric gene may be an important determinant of diastolic dysfunction in FHC.

IX.P17.

Cardiomyopathy-causing mutations in cardiac thin filament regulatory proteins acutely affect Ca^{2+}-buffering and Ca^{2+}-dependent signalling in situ

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Hypertrophic cardiomyopathy (HCM) is caused by mutations in thin filament regulatory proteins that confer distinct primary alterations of cardiac contractility. We believe that altered Ca^{2+}-buffering by mutant thin filaments leads to altered Ca^{2+}-handling and, via Ca^{2+}-dependent signalling pathways, contributes to the pathogenesis of disease. We are studying the in situ effect on Ca^{2+}-flux of a HCM causing mutations in human cardiac troponin T (TnT) (R92Q), troponin I (TnI) (R145G) and α-tropomyosin (αTM) (D175N) by adenoviral mediated expression in adult guinea pig cardiomyocytes. Western blot and immunolocalisation analysis of infected cells showed that recombinant FLAG tagged protein was found at the I band and comprised ~50 % of the total TnT, TnI or αTM, 48 h after infection. Simultaneous measurement of unloaded sarcomere-shortening and Ca^{2+}-transients using fura-2 loading, showed the HCM mutations had a significant decrease in the basal sarcomere length coupled with an increase in the diastolic Ca^{2+}-concentration. The mechanism of alterations to EC-coupling was investigated using tetracaine and caffeine challenging combined with simultaneous patch clamping. HCM mutant cells displayed reduced SR load (~1.4-fold), slowed NCX calcium extrusion (~2.5-fold), increased SERCA2 activity (~3-fold), increased ryanodine receptor leak (~5-fold) and increased calcium buffering (~4-fold). Ca^{2+}-

dependent signalling cascades were assessed by observing a ~2.5-fold increase in nuclear translocation of the transcription factor NFAT by immunofluorescence, when the cells were chronically paced in culture. These data strongly implicate Ca^{2+}-buffering as a result of increased myofilament calcium sensitivity as a driving mechanism of remodelling in the pathological disease state of HCM.

IX.P18.

Low expression of the troponin T mutation K280N increases myofilament Ca^{2+}-sensitivity in human cardiomyocytes

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Background Transgenic mice expressing mutant troponin T (cTnT) indicate that mutation-induced myofilament Ca^{2+}-sensitization increases the incidence of cardiac arrhythmias in hypertrophic cardiomyopathy (HCM). We investigated wether the homozygous cTnT K280N mutation increases myofilament Ca^{2+}-sensitivity in the human heart.

Methods Force measurements were performed in single cardiomyocytes isolated from a HCM patient with a homozygous K280N mutation (K280N) and donor myocardium. To discriminate between mutation-induced and disease-related effects on Ca^{2+}-sensitivity, cardiomyocytes were treated with alkaline phosphatase (AP) and protein kinase A (PKA). Exchange experiments with 0.25, 0.5 and 1 mg/mL recombinant human wild-type troponin (Tn_{wt}) were performed in K280 N cells yielding 62, 78 and 86 % of Tn_{wt} exchange, respectively. In addition, donor cardiomyocytes were exchanged with 45 % recombinant K280 N mutant protein.

Results Myofilament Ca^{2+}-sensitivity was higher in K280 N cells compared to donor. K280 N myocardium showed lower cTnT and higher myosin light chain 2 phosphorylation compared to donor, while phosphorylation of the PKA-target proteins was similar. Both AP- and PKA-treatment did not eliminate the difference in Ca^{2+}-sensititivity between K280 N and donor. K280N cells exchanged with various concentrations of Tn_{wt} showed a higher myofilament Ca^{2+}-sensititivity compared to donor that did not change after PKA treatment. Similarly, exchange of donor cells with 45 % recombinant K280 N increased myofilament Ca^{2+}-sensitivity, which was not rescued to donor levels after PKA-treatment.

Conclusions In human myocardium the homozygous cTnT mutation K280N is associated with intrinsically increased myofilament Ca^{2+}-sensitivity and reduced cTnT phosphorylation. Low (<20 %) mutant-protein expression is sufficient to increase Ca^{2+}-sensitivity of the myofilaments and may be sufficient to trigger disease onset in heterozygous K280N carriers.

IX.P19.

Ca^{2+}-binding to both regulatory sites of skeletal troponin C is required for inducing the regulatory switch of troponin I

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Skeletal troponin C contains two regulatory Ca^{2+}-binding sites. Whether binding of one or two Ca^{2+}-ions are required for enabling the regulatory switching of skeletal troponin I (sTnI) from actin to troponin C is unknown. We probed the kinetics of this switch by incorporating skeletal troponin complex labelled at Cys134 of sTnI into rabbit psoas myofibrils and rapidly mixing the myofibrils in a stopped flow apparatus with different [Ca^{2+}]. The fluorescence transients were biphasic yielding [Ca^{2+}]-dependent rate constants for both phases. Successful simulation of the biphasic fluorescence transients requires a minimum model consisting of two conformational changes of cTnI in which the first change already can occur upon binding of one Ca^{2+}-ion whereas the second change only can occur upon binding of both Ca^{2+}-ions to troponin C. This model is different to the more simple model for describing the switch kinetics observed with cardiac troponin that contains only a single regulatory Ca^{2+}-binding site on its troponin C subunit. The implications of these findings for the cooperativity and the speed of thin filament activation in skeletal and cardiac muscle will be discussed.

IX.P20.

Phosphorylation of cardiac troponin I is altered through expression of HCM-mutant cTnI-R145G

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Hypertrophic cardiomyopathy (HCM) is a disease primarily linked to mutations in genes of sarcomeric proteins. The amino acid exchange R145G in the inhibitory subunit (I) of cardiac troponin (cTn) results in impaired relaxation of myofibrils and enhanced risk for sudden cardiac death. Overexpression of human (h) cTnI-R145G in adult rat cardiomyocytes leads to suppression of contractile parameters as well as elongated calcium transients. Suppressed contractile dynamics were also present upon $beta_2$- but not $beta_1$-adrenoceptor (AR) stimulation, where defects were compensated upon stimulation. Western blot analysis of the phosphorylation status of protein kinase A (PKA)-sites Ser22,23 tended to be reduced, whereas global phosphorylation state of cTnI was similar in hcTnI-wt and -R145G overexpressing cells. This indicates a modified phosphorylation pattern of cTnI due to the mutation. Preliminary analysis of phosphoisoforms using phosphate affinity SDS-PAGE indeed appears to show additional phosphorylation sites in cTnI-R145G before and after $beta_1$- and $beta_2$-AR stimulation. The suppression of contractile parameters by hcTnI-R145G could largely be reversed by exchanging both PKA-sites with Asp, whereas beta-AR stimulation of cells expressing this pseudo-phosphorylated mutant elevated parameters still further. This might be due to phosporylation of additional sites on sarcomeric proteins. A compensating effect through other proteins could also be likely, although no changes in the phosphorylation of myosin light chain 2, myosin binding protein C or cTnT were found using Pro-Q Diamond or 2D gel electrophoresis. In conclusion our findings indicate that alteration in cTnI phosphorylation due to cTnI-R145G expression in cardiomyocytes contributes to an altered beta-adrenergic response.

IX.P21.

Steady state and short-term interval force relationship in ventricular trabeculae from Δ160E and E163R cTnT mutant mice

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Mutations in cardiac troponin T (cTnT) lead to hypertrophic cardiomyopathy, associated with a large spectrum of clinical phenotypes. Here we aim to compare the mechanical behavior of a the well-established cTnT transgenic mouse model (Δ160E, 35 % mutant protein expressing line) to a novel mouse line carrying E163R mutation, expressing 50 % mutant protein.

Methods Left and right ventricular trabeculae were dissected from non-transgenic wild type (WT) and heterozygous (Δ160E or E163R) mouse hearts and mounted isometrically to record twitch tension.

Results While Δ160E hearts showed smaller ventricles and mildly dilated atria compared to sibling WT hearts, E163R hearts showed neither hypertrophic/restrictive phenotype nor ventricular dilation. Isometric twitch tension at 2 Hz was comparable in the three groups. The positive inotropic response to high stimulation rates was hampered in Δ160E, but preserved in E163R mice. The kinetics of force development and relaxation was prolonged in Δ160E and, to a larger extent, in E163R mice, while rate dependent adaptation of twitch duration was always preserved. In both lines, mechanical refractoriness was shorter and restitution of isometric tension was significantly faster than in WT, while maximum post-rest potentiation tended to be lower. Preliminary intracellular Ca^{2+}-transient measurements show changes that are consistent with those of twitch tension.

Conclusions Steady-state contractions are prolonged in Δ160E and E163R mice. In both lines, the short-term interval force relationship, i.e. mechanical restitution and post-rest potentiation, show changes that may be compatible with an increased RyR2 opening probability.

IX.P22.

R299Q AMPK γ2 knock-in mouse model exhibits increased Ca^{2+}-sensitivity of cardiac activation and enhanced cardiomyocyte contractility

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AMP-activated protein kinase (AMPK) is an evolutionarily conserved, heterotrimeric enzyme involved in energy balance regulation and cell signalling. Mutations in the gene encoding the γ2 subunit of AMPK (*PRKAG2*) cause a cardiomyopathy characterized by ventricular hypertrophy, glycogen accumulation, contractile dysfunction, and aberrant electrical conduction. Given our recent finding that cardiac troponin I (cTnI) is phosphorylated by AMPK at S150 and AMPK activation enhances cardiomyocyte contractility, we hypothesise that changes at the myofilament level may contribute to disease pathogenesis. To test this, we have used a mouse model possessing an AMPK γ2 mutation (R299Q knock-in) and measured fibre mechanics and cardiomyocyte contractility using heterozygous and homozygous male mice, along with littermate wild type controls, between 6 and 8 weeks of age (prior to significant glycogen accumulation). In demembranated trabeculae, we observed increased Ca^{2+}-sensitivity of force production and reduced cooperativity of activation in mutant groups. Intact cardiomyocytes isolated from mutant hearts exhibited increased contractility, increased resting sarcomere length, reduced resting [Ca^{2+}], and altered stimulation frequency sensitivity. Biochemical analyses indicated reduced phosphorylation of cTnI at the 23/24 site as well as reduced phosphorylation of phospholamban in the mutant groups. We did not detect phosphorylation at cTnI S150 in any sample. These results suggest AMPK γ2 mutations alter cardiac contractile mechanics via modified PKA activity. These alterations were observed irrespective of glycogen accumulation and therefore support the hypothesis that the mutations cause a primary disorder of the myocardium.

IX.P23.

DCM-causing mutation E361G in actin slows myofibril relaxation kinetics and uncouples myofibril Ca^{2+}-sensitivity from protein phosphorylation

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Experiments using the in vitro motility assay have shown that the myofilament Ca^{2+}-sensitivity is modulated by phosphorylation of troponin I and that the DCM-causing mutation *ACTC* E361G uncouples this relationship. Here, we determine whether this also happens in heart muscle myofibrils producing isometric force. We know that TnI and MyBP-C phosphorylation levels are high in normal mouse heart (1.1 mol Pi/mol TnI). To reduce the phosphorylation level of TnI and MyBP-C, mice were injected with a high dose of propranolol which reduced of both TnI bisphosphorylation and MyBP-C phosphorylation by at least 95 %. Myofibrils were isolated from propranolol-treated and untreated mouse hearts. Then we measured contraction in single myofibrils with a Ca^{2+}-jump protocol using a range of Ca^{2+}-concentrations. The Ca^{2+}-sensitivity of isometric force and the kinetics of tension development and relaxation for the *ACTC* E361G TG mouse myofibrils were compared with WT. Modulation of the Ca^{2+}-sensitivity by changes in sarcomere length and by the Ca^{2+}-sensitizer EMD 57033 was further investigated.

The maximum isometric force and the tension development rate was the same for phosphorylated and dephosphorylated WT and ACTC E361G mouse myofibrils. The relaxation rate was reduced 1.3-fold for the ACTC E361G TG compared to WT. The Ca^{2+}-sensitivity of isometric force was increased 1.4-fold when WT myofibrils were dephosphorylated. In contrast the ACTC E361G Ca^{2+}-sensitivity was not altered by dephosphorylation. Comparing dephosphorylated WT vs WT, the relaxation rate was 1.3-fold slower but dephosphorylation did not change the kinetics of E361G myofibril. Thus the ACTC E361G mutation uncoupled Ca^{2+}-sensitivity and lusitropy from the level of TnI and MyBP-C phosphorylation in intact myofibrils.

IX.P24.

Optical trapping directly reveals a threefold increase in head stiffness by the FHC-related R723G mutation in the β-cardiac myosin heavy chain

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Missense mutations in the β-cardiac myosin heavy chain (β-MyHC) are the most frequent cause of Familial Hypertrophic Cardiomyopathy (FHC). For these missense mutations mutant and wildtype β-MyHC are co-incorporated into the contractile apparatus, and the direct functional effects of such mutations presumably trigger development of the FHC-phenotype. In M. soleus biopsies we found the converter mutation R723G to increase force generation due to increased stiffness of the myosin head. Quantification of this increase from fiber studies, however, was complicated by filament compliance, as well as non-equal and variable co-expression of mutant and wildtype protein in the tissue samples.
Now we directly determined stiffness of individual myosin heads isolated from M. soleus tissue samples of affected patients using the three-bead optical trapping assay. Since each assay contained mutant and wildtype head domains stiffness of individual heads with/without the R723G mutation could directly be compared. We determined head stiffness by imposing triangular stage displacements and related this to observed bead displacements. We found two populations of head domains, one with a head stiffness of 0.39 + 0.24 pN/nm the other with an about threefold higher stiffness. The low value is close to our previous optical trapping with β-MyHC (0.38 + 0.06 pN/nm), the about threefold increase in head stiffness agrees well with our previous estimate from rigor stiffness in fibers with the same mutation while the absolute value of head stiffness estimated for fibers was about 25 % lower than determined here.

IX.P25.

The DCM-causing mutation actc E361G blunts the response to adrenergic stimulation and predisposes towards heart failure under chronic stress in a transgenic mouse

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We have investigated a number of dilated cardiomyopathy-causing contractile protein mutations in vitro and found a distinctive uncoupling of changes in myofilament Ca^{2+}-sensitivity from the level of troponin I phosphorylation. We propose this "uncoupling" constitutes the causal mechanism in DCM caused by sarcomeric protein mutations. We hypothesise that this uncoupling leads to a blunted response to adrenergic stimulation and thus loss of cardiac reserve, with consequent heart failure under stress.
To examine our hypothesis we used a pressure–volume catheter in vivo to record pressure–volume loops from transgenic (TG) mice carrying a known DCM-causing actin mutation (ACTC E361G) and their non-transgenic (NTG) littermates. We show that the dobutamine-induced increase in cardiac output, ejection fraction and heart rate is significantly smaller in TG mice than NTG controls. To examine the effect of chronic stress we also studied pressure–volume loops in TG and NTG mice exposed to 4-week angiotensin II infusion. Significantly, the reduced cardiac reserve predisposed TG mice to heart failure, characterised by significantly lower cardiac output, ejection fraction and speed of contraction (dP/dt_{max}). Work is in progress to investigate the consequences of uncoupling in intact papillary muscles isolated from ACTC E361G and NTG mice.
In conclusion, we show that a DCM-causing sarcomeric protein mutation that causes uncoupling of Ca^{2+}-sensitivity from the level of TnI phosphorylation with a consequent blunted response to adrenergic stimulation. Given the physiological sequelae we observed we propose that this uncoupling plays a major role in the pathogenesis of DCM caused by sarcomeric protein mutations.

IX.P26.

Increased tension cost in hypertrophic cardiomyopathy depends on the location of the mutation in the gene encoding myosin heavy chain

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Introduction Familial hypertrophic cardiomyopathy (HCM) is frequently caused by missense mutations in the *MYH7* gene encoding β-myosin heavy chain (β-MHC). Disease onset and severity varies even among patients harboring mutations in the same gene. This might be explained by the location of the mutation in the encoded protein and its functional consequences. We studied if missense mutations in different regions of β-MHC exert diverse effects on energetic cost of tension development.
Methods Tension cost (TC) was determined by simultaneous measurements of tension generation and ATP-utilization in Triton-permeabilized cardiac muscle strips of HCM patients with mutations in the S1 domain (R403Q, n = 3; V606 M, n = 1) and rod (T1377 M, n = 1) of β-MHC. Strips of sarcomere mutation-negative patients (HCM_{smn}; n = 5) were used as controls. TC was calculated as the rate of ATP-utilization divided by the amount of tension generated. In addition, the fraction of mutated mRNA was analyzed using reverse transcription-PCR and subsequent mutation specific restriction digests.
Results Tension was significantly reduced in HCM_{R403Q} while ATP utilization was similar. This resulted in significantly higher TC in HCM_{R403Q} (Figure). The fractions of mutated mRNA ranged from 35.2 to 45.6 % in HCM_{R403Q}, 35.1 % in HCM_{V606} and 52.3 % HCM_{T1377M}. Mutated mRNA content did not correlate with TC as the highest TC (5.6 μmol/Ls^{-1}) was found in a HCM_{R403Q} sample expressing the lowest level of mutated mRNA (35.2 %).
Conclusion Our data indicate that a relatively low level of mutated β-MHC is sufficient to increase TC in patients with the R403Q missense mutation. Using human HCM tissue we provide evidence that high tension cost as a potential HCM disease mechanism depends on *MYH7* mutation location.

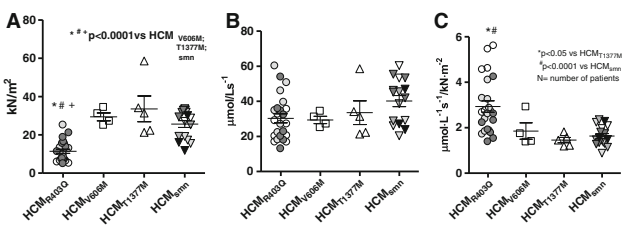

Figure **a** Tension, **b** ATP utilization and **c** tension cost

POSTER SESSION X Regulation of cardiac muscle contraction in health and disease

X.P1.

Molecular model confirms differences in helical pitch of peptides in subfragment S2 of cardiac myosin isoforms and mutations

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We reported in a previous work that the measured helical pitch of peptide chains of cardiac myosin in relaxed tissue by polarization second harmonic generation (PSHG) were significantly different for 1-year adult rabbits and young rats1. We hypothesized that these changes were produced by differences in the distribution of cardiac myosin isoforms, MYH7 and MYH6. Previous studies have shown that young rats have characteristic 90 % MYH6 and 10 % MYH7 whereas 1-year old rabbit achieve an almost inverse relationship with 5 and 95 %, respectively. In order to evaluate this, we downloaded the public sequences of both, MYH6 Rattus norvegicus (MYH6-rat), NCBI, NP_058935.2, and MYH7 Oryctolagus cuniculus (MYH7-rab), UniProtKB/Swiss-Prot: P04461.1, and generated a specific 3D molecular model of S2 subsegment, which contains the elementary unit of PSHG activity, for each sequence based on reported crystallographic results of human MYH7 (PDB:2FXM). PSHG reported a median angle of 71.8° for MYH7-rab and 66.1° for MYH6-rat. Current molecular computations resulted in 72.4° and 66.9°, respectively, showing agreement. We also analyzed subfragments S2 of human MYH7 for control and E924K mutation (related to hypertrophic cardiomyopathy) and found different angles of 67.1° and 73.6°, respectively, which can be detected by current PSHG schemes. In conclusion, this analysis further supports the hypothesis that PSHG is able to measure subtle differences in molecular architecture that are available only thorugh much more complex techniques such as crystallography. Furthermore, this technique is open to in vivo experiments and has the potential to provide dynamic information during contraction in healthy and diseased hearts.

X.P2.

The functional significance of the proline–alanine and C1 regions of cMyBP–C in the timing of cardiac contraction in vivo

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A role is emerging for cardiac myosin binding protein C (cMyBP-C) in regulating speed of cardiac contraction, but neither the exact region nor the mechanism(s) that underlie this function are well understood. Previous in vitro studies indicated that the proline-alanine (p/a) rich region and C1 domain of cMyBP-C can promote cross bridge activation.

Mice lacking the p/a and C1 region (p/a-C1$^{-/-}$ mice) do not show signs of cardiac hypertrophy, as evident from the heart/body weight ratio, left ventricular diameter and wall thickness, fractional shortening and ejection fraction all being comparable to wild type mice. However, force measurements in skinned cardiomyocytes from p/a-C1$^{-/-}$ mice demonstrated a modestly increased speed of force redevelopment (k_{tr}) at submaximal Ca^{2+}-concentrations by 10-20 %. No differences between groups were observed in maximal or passive force development or Ca^{2+}-sensitivity. Troponin I and cMyBP-C phosphorylation was also not different between groups, indicating a normal β–adrenergic system in p/a-C1$^{-/-}$ mice.

An increase in the speed of force redevelopment especially at submaximal Ca^{2+}-concentrations in p/a–C1$^{-/-}$ mice could indicate a role for the p/a-C1 region in the regulation of cross bridge cycling speed and timing of cardiac contraction. In vivo hemodynamic recordings will clarify the relevance of such a mechanism and the role of the p/a-C1 region to the function of the heart as a whole organ.

X.P3.

Numbers of intrinsic cardiac adrenergic cells in rat heart: effect of hypertrophy

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The efficiency of hypertrophied myocardium is reduced. Upregulation of oxygen consuming enzymes, like monoamine oxidase A (MAO-A), reduces efficiency and contributes to ROS production. Inhibition of MAO-A in isolated papillary muscle reduced oxygen consumption, suggesting endogenous catecholamine release. We hypothesize that intrinsic cardiac adrenergic (ICA) cells, which predominantly produce noradrenalin, are involved. We counted ICA cells in sections of monocrotaline (MCT, 60 mg/kg) administered male Wistar rats by tyrosine hydroxylase immunohistochemistry. Two-way ANOVA and post hoc tests with Bonferroni correction were performed. The number (mean ± SEM) of ICA cells in controls ($n = 3$) was 207 ± 29 cells/μl in the right free wall, 264 ± 19 cells/μl in the right side of the septum, 162 ± 51 cells/μl in the left side of the septum, and 204 ± 16 cells/μl in the left free wall. ICA cell density in the left and right side of the septum differed ($p < 0.05$). MCT rats developed pulmonary hypertension and right ventricular hypertrophy. The number of ICA cells in these hearts ($n = 5$–7) was 130 ± 11 cells/μl in the right free wall, 238 ± 13 cells/μl in the right side of the septum, 209 ± 20 cells/μl in the left side of the septum, and 231 ± 23 cells/μl in the left free wall. ICA cell density in the right free wall was smaller than in other areas of the hearts ($p < 0.05$). The interaction of treatment and location was not significant ($p = 0.075$). The decrease in ICA cell density in the right free wall can be explained by hypertrophy of cardiomyocytes. We conclude that ICA cell density is high and that the number of ICA cells does not change in hypertrophied rat myocardium.

X.P4.

Does inorganic phosphate inhibit cardiac myofibrillar force production at 37 °C?

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Introduction In the healthy heart, inorganic phosphate (P_i) is present at a concentration of few millimolar, rising to 20 mM or more during

cardiac ischaemia. Studies with skinned cardiac preparations at 15–23 °C have shown that Pi reduces force generation. However, in skinned skeletal fibres a similar inhibitory action of Pi at low temperatures has been reported to be reduced or abolished at temperatures closer to physiological. We therefore compared the effects of P_i on force development in skinned cardiomyocytes at 15 and 37 °C.

Method Permeabilised cardiomyocytes from human ventricular myocardium (from healthy, unused donor hearts) were maximally activated (30 μM free Ca^{2+}) in the presence of P_i (0–20 mM added). A rapid 1 % stretch was applied during isometric force development to additionally measure crossbridge kinetics.

Results Maximum Ca^{2+} activated force decreased exponentially as $[P_i]$ was raised. The sensitivity to P_i was similar for the two temperatures, e.g. 20 mM added P_i decreased force by ~87 % at 15 °C and ~78 % ($n = 4$) at 37 °C ($p = 0.74$). The rates of stretch-induced cross-bridge detachment (k_{rel}) and the subsequent force re-development (k_{df}) increased exponentially as a function of Pi. Surprisingly, these two kinetic parameters were not significantly faster at the higher temperature.

Conclusion These findings suggest that, in single cardiomyocytes, the effect of P_i on force development is substantial at both 15 and 37 °C. The inhibitory effect of P_i on myofibrillar force production is therefore likely to contribute to the contractile dysfunction induced by cardiac ischaemia in vivo.

X.P5.

Cardiac and skeletal muscle protein isolation from zebrafish for in vitro motility assay

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The understanding of mutations of motor or regulatory muscle proteins leading to cardiac insufficiency and skeletal muscle deficiency helps to design better strategies to overcome such abnormalities. Zebrafish has been used as an animal model for diverse human cardiac diseases; however, most studies focused on molecular and biochemical aspects, neglecting the function, especially the relevance of motor and regulatory proteins in force production. The interaction and mechanical properties of motor proteins can be investigated by in vitro motility assay (IVMA), in which the maximum sliding speed of labeled actin filaments moved by working strokes of the heads of immobilized myosin molecules under optimal ATP-concentration can be measured. So far IVMA has not been used to study acto-myosin from zebrafish cardiac and skeletal muscle.

We established isolation protocols for skeletal and cardiac myosin and polymerizable actin from zebrafish. Cardiac and skeletal motor and regulatory muscle proteins from adult wild type zebrafish extracts were confirmed by SDS-PAGE and western blot and their activity by IVMA. Functional cardiac myosin was extracted from hearts of wildtype (TÜ AB) and heterozygous cardiac essential myosin light chain-1 mutant (*lazy susan*) zebrafish directly into the IVMA flow-cells, after swimming endurance was observed in a custom-made counterflow swimming chamber. These successful isolation protocols for muscle proteins from zebrafish allowed us to analyze instantaneous speeds and travelled distances over time of actin filaments sliding on skeletal and cardiac zebrafish myosin. Our results suggest that this counterflow swimming protocol enhances any phenotype differences between wild type and mutants.

X.P6.

Effects of cardiac myosin binding protein-C on the mechanical interaction of cardiac isomyosins V1 and V3 with actin filament in an in vitro motility assay and optical tweezers

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Modulatory role of whole cardiac myosin binding protein-C (cMyBP-C) in regulation of cardiac muscle contractility was studied in the in vitro motility assay with rabbit cardiac myosin isoforms V1 and V3. The effects of cMyBP-C on the interaction of single cardiac myosin molecule with actin were tested by the optical tweezers method.

Using optical trap we found that the addition of cMyBP-C did not affect appreciably mechanical characteristics of the actin-myosin interaction (step size and force developed by myosin head) for both isomyosins. As for kinetic characteristics of this interaction, the addition of cMyBP-C affected only the duration of force developed by V1.

We found that addition of cMyBP-C at physiological proportion (1:5 molar ratio of cMyBP-C/myosin) to the motility assay led to a decrease in the sliding velocity of regulated thin filaments at maximal calcium ($pCa = 4$) for both isomyosins. The presence of cMyBP-C not shifted calcium sensitivity (assessed as pCa_{50}) of "pCa–velocity" relationships for both isomyosins. The Hill coefficient of "pCa–velocity" relationship decreased for V3, but did not change in the case of V1.

X.P7

Patients with lymphocytic myocarditis have increased lymphocytes in the skeletal muscle: a new diagnostic tool

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Background Lymphocytic myocarditis (LM) is difficult to diagnose and presently relies largely on (immuno)histologic analysis of endomyocardial biopsies. Several studies indicate that cardiac inflammation is linked to local immune-responses in the skeletal muscle. In this study, we have quantitatively analyzed the presence of inflammatory cells in quadriceps skeletal muscle of LM patients and its potential as a new diagnostic tool in LM.

Methods From patients that were diagnosed post-mortem with LM ($n = 21$) and control patients ($n = 9$), skeletal muscle tissue was stained immunohistochemically for lymphocytes (CD45), macrophages (CD68), neutrophilic granulocytes (MPO), lymphocyte subsets (CD3, CD4, CD8 and CD20) and were subsequently quantified. Lymphocytes were also quantified in skeletal muscle in non-infectious inflammatory diseases of the heart, namely stress-

induced myocarditis (n = 6) and acute myocardial infarction (AMI, n = 26). Finally as a proof of concept study the same analysis was performed in a mouse model of acute coxsackievirus B3-induced myocarditis.

Findings The number of lymphocytes (CD45), T-lymphocytes (CD3) and cytotoxic T-lymphocytes (CD8) were significantly increased in skeletal muscle of LM patients, compared with control patients. Also in patients with chronic phase AMI and in mice with acute coxsackievirus B3-induced myocarditis, a significant increase in lymphocytes in skeletal muscle tissue was found. Notably, viral RNA was detected in the skeletal muscle of all but one mice with coxsackievirus B3-induced myocarditis, while no viral RNA was detected in skeletal muscle of control mice.

Interpretation This study demonstrates that the number of lymphocytes in skeletal muscle is increased in patients diagnosed with LM and in mice with coxsackievirus B3-induced myocarditis, coinciding with viral RNA in the skeletal muscle in mice, and demonstrates that lymphocytes in skeletal muscle biopsies facilitates diagnosing of LM.

X.P8.

PDE3 inhibition by levosimendan is sufficient to account for its inotropic effect in failing human heart

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Background and purpose Levosimendan is known as a calcium-sensitiser, although it is also known to inhibit phosphodiesterase (PDE) 3. We aimed to isolate each component and estimate their contribution to increased contractility.

Experimental approach Contractile force was measured in electrically stimulated ventricular strips from explanted failing human hearts and left ventricular strips from normal male Wistar rats. PDE activity was measured in a two-step PDE activity assay on failing human ventricle.

Key results Levosimendan revealed a positive inotropic response (PIE) reaching maximum at 10^{-5} M on ventricular strips from failing human hearts. In the presence of the selective PDE3 inhibitor cilostamide, the PIE of levosimendan was abolished. During treatment with a PDE4 inhibitor and a supra-threshold concentration of isoproterenol, levosimendan generated an amplified inotropic response. This response was reversed by β-adrenoceptor blockade and undetectable in strips pre-treated with cilostamide. Levosimendan (10^{-6} M) increased the potency of β-adrenergic stimulation (isoproterenol) by 0.5 log units in failing human myocardium. In the presence of cilostamide, levosimendan failed to potentiate isoproterenol. Every inotropic response to levosimendan was associated with a lusitropic response. Levosimendan did not shift the concentration–response curve to calcium in rat ventricular strips, as opposed to the calcium sensitiser EMD57033. PDE activity assays confirmed that levosimendan inhibited PDE3 with similar potency as cilostamide.

Conclusions and implications Our results indicate that the PDE3-inhibitory property of levosimendan is sufficient to account for its inotropic effect, leaving a minor if any effect to a calcium-sensitising component.

X.P9.

Inactivation of inhibitory G protein (G_i) regulates receptor independent adenylyl cyclase activity increasing ventricular contractility

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Background and purpose We hypothesize that G_i regulates adenylyl cyclase (AC) activity independent of receptor activation, since β_1 and β_2-adrenergic receptor (βAR)-evoked inotropic responses are modified by pertussis toxin (PTX) inactivation of G_i, despite only β_2AR dually coupling with G_s and G_i.

Experimental approach We utilized the unique properties of fenoterol stereoisomers (β_2AR agonist): (R,R) activating only G_s, (R,S) activating G_s and G_i. Receptor-independent AC activity was studied using forskolin and phosphodiesterase 3,4 (PDE3,4) inhibitors. Contractility and cAMP accumulation was measured in rat left ventricular myocardium with or without PTX pre-treatment to inactivate G_i.

Key results PTX-treatment amplified both fenoterol stereoisomers-evoked cAMP accumulation ~2-fold. Without PTX, both stereoisomers evoked small inotropic responses. In PTX-treated rats, the maximal inotropic response to both stereoisomers was significantly amplified. The PTX enhancement of the G_s-selective (RR)-fenoterol inotropic response suggests that G_i regulates AC activity independent of receptor activation and dual coupling to G_s and G_i. Consistent with this hypothesis, concentration–response curves to forskolin were also potentiated by PTX treatment. PDE3,4 inhibition in non-PTX-treated, or removal of constitutive muscarinic receptor activation of G_i with atropine in PTX-treated tissue had no effect on basal contractility. However, PDE3,4 inhibition alone increased basal cAMP production and evoked a large inotropic response only in PTX-treated tissue. Together, these data indicate that G_i exerts intrinsic receptor-independent inhibitory activity upon AC.

Conclusions and implications G_i regulates translation of cAMP production to functional responsiveness independent of receptor activation. We hypothesize that PTX treatment shifts the balance of constitutive G_i and G_s activity upon AC, resulting in potentiation of all cAMP-mediated inotropic effects.

X.P10.

Effects of nitric oxide (NO) on Ca^{2+}-transients recorded as fluo-4 signal from electrically driven in isolated murine cardiomyocytes

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In cardiomyocytes unregulated Ca^{2+}-release and an uncontrolled increase of Ca^{2+}-concentration can induce severe contractile dysfunction, arrhythmias, high blood pressure, protein degradation and cell death. Nitric oxide (NO) has been shown to have modulating effects on molecules involved in Ca^{2+}-handling, as well as regulation of contractility, mitochondrial oxygen metabolism and glucose. The mutation on synthases of NO (NOS) induces abnormal electrophysiological parameters seen in the electrocardiogram compared to normal cardiomyocytes, which are restored with either transgenic overexpression of NOS or NO donors. However, lacunae exist regarding the effect of NO on biophysical parameters of temporal properties of Ca^{2+}-transients within isolated murine cardiomyocytes.

Ca^{2+}-transients were recorded from adult wild-type mouse isolated cardiomyocytes loaded with Fluo-4 (5 μM) and electrically stimulated (1 Hz, 18 V, 10 ms). Effects of NO were investigated using spontaneous NO donor (SNAP, 100 μM), NOS blocker (L-NAME, 5 mM) or guanylate cyclase blocker ODQ (10 μM). Ca^{2+}-transients were modified using CPA (5 μM) and caffeine (2 mM). The following Ca^{2+}-transients parameters were measured: width (ms), height (V), time to peak (ms), peak area (V*ms), tau (s) and slope (V/s).
SNAP decreased the inhibitory effect of CPA on $SERCA_2$ thereby increasing Ca^{2+}-sequestration into SR. Moreover, SNAP modified the caffeine-mediated Ca^{2+}-release from SR.
These results support previous reports, which showed that NO modify $SERCA_2$-regulation. Moreover, gained results suggest that NO act on cardiac isoform of ryanodine receptor (RyR_2) and on $SERCA_2$-regulation, probably via guanylate cyclic dependent pathway.

X.P11.

Ca^{2+}-sensitizing effects of OR-2828 in permeabilized cardiomyocytes

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OR-2828, a potential calcium-senzitizer drug with a molecular structure similar to that of levosimendan have a dextrorotatory (R-OR-2828) and a levorotatory (L-OR-2828) stereoisomer. The aim of this study was to characterize the hypothetical Ca^{2+}-sensitizer effects of these optical isomers in isolated permeabilized cardiomyocytes.
Force measurements were performed in myocyte-sized preparations derived from left ventricular preparations of guinea pigs, as well as from failing human hearts with dilated cardiomyopathy. Isolated and permeabilized cardiomyocytes, attached between a force transducer and an electromagnetic motor were exposed to test solutions with increasing concentrations of the two optical isomers of OR-2828 (between 10nM and 100 μM) to test their concentration dependencies on force production and on its Ca^{2+}-sensitivity.
10 μM L-OR-2828 significantly ($p < 0.05$) increased Ca^{2+}-sensitivity of force production in myocyte-sized preparations derived from guinea pigs hearts (pCa_{50}: 5.76, vs. the drug free control of 5.69; $n = 8$) and from failing human hearts with dilated cardiomyopathy (pCa_{50}: 6.18, vs. a drug free control of: 6.07; $n = 14$). The EC_{50} value for L-OR-2828 effect was 2.44 μM in guinea pig hearts. L-OR-2828 had no effect on force generation at maximal Ca^{2+}-concentration (pCa 4.75) and on Ca^{2+}-independent passive force. R-OR-2828 did not affect Ca^{2+}-sensitivity of force production in cardiomyocytes of guinea pig hearts.
Our data illustrate L-OR-2828 as a positive inotropic agent, exerting its cardiotonic effect similarly to levosimendan via a Ca^{2+}-sensitizing mechanism.

X.P12.

Study of effects of tropomyosin isoforms on the regulation of actin–myosin interaction in myocardium with in vitro motility assay

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Interaction of myosin with actin in striated muscle is controlled by Ca^{2+} via thin filament associated proteins: troponin and tropomyosin. In cardiac muscle there is a whole pattern of myosin and tropomyosin isoforms. Regulatory effect of tropomyosin isoforms (with different content of α- and β-chains) on acto-myosin interaction in cardiac muscle was studied in the in vitro motility assay with rabbit cardiac isomyosins V1 and V3. We estimated the dependence of both the sliding velocity of actin/actin–tropomyosin filaments and "pCa-velocity" relationship on myosin and tropomyosin isoforms.
As we found tropomyosins of different content of α- and β-chains being added to actin filament affect the sliding velocity of filaments in different ways. On the other hand the velocity of filaments with the same tropomyosins depends on heavy chains isoforms of cardiac myosin.
The sliding velocity of regulated thin filaments at maximal calcium ($pCa = 4$) also depends on both myosin and tropomyosin isoforms. The Hill coefficient of "pCa-velocity" relationship did not depend on both tropomyosin and myosin isoforms. On the contrary calcium sensitivity (taken as pCa_{50}) was markedly different for tropomyosin isoforms in the case of V3 myosin and did not vary for V1 isoform.

X.P13.

3D reconstruction of subsarcolemmal sarcoplasmic reticulum of sheep cardiac myocytes using serial block face SEM

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The ultrastructure of the sarcoplasmic reticulum (SR) is related to its function yet there is currently little high resolution data available. We have applied the novel technique of serial block face scanning electron microscopy (sbfSEM) to investigate the SR organisation within a cardiomyocyte. Samples of sheep left ventricle were processed for routine electron microscopy modified to specifically stain the SR. Resin blocks were positioned in an FEI Quanta 250 FEG SEM equipped with a Gatan 3View system, a configuration with a microtome in situ which slices through the block. An image of the block face was taken after the removal of each slice thereby generating volumetric data through the tissue with a resolution of ~15 nm at the block surface and ~50 nm entering into the block. Images were analysed and segmented in Fiji or IMOD. 3D reconstructions revealed several new features of the SR architecture, finding regions running along and in contact with the mitochondria, an organisation which may underlie a critical role in local Ca^{2+} transfer between the two organelles. Our data also revealed that the SR forms a continuous network crossing the Z-lines linking the entire cell. Moreover, we found that the SR forms multiple specialised junctions (jSR) with the sarcolemma as well as with the transverse tubules. Subsarcolemmal jSR is consistent with a functional role for spark generation in cardiomyocytes around the edges of the cell. A similar organisation would be relevant to myocytes lacking a developed t-system like atrial myocytes of small mammals.
We acknowledge and thank the British Heart Foundation for funding this work (AK and AWT; RG/11/2/28701).

X.P14.

Molecular determinants of increased right ventricular cardiomyocyte stiffness

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Introduction Patients with pulmonary arterial hypertension (PAH) develop severe right heart failure with impaired diastolic function. Right ventricular (RV) sarcomere stiffness and Ca^{2+}-sensitivity are increased in PAH-patients. The aim of this study is to investigate whether changes in sarcomere protein phosphorylation and/or titin isoform expression explain the observed changes in RV sarcomere function in PAH-patients.

Methods Human RV tissue was obtained from PAH-patients after heart–lung transplantation and from non-failing donors. To assess if high passive stiffness is due to changes in titin composition, Titin isoform expression and phosphorylation of protein kinase A (PKA) (Ser469) and protein kinase C (PKC) (Ser26&170) sites were assessed by 1 %-Agarose gel electrophoresis and western blot analysis. In addition, RV cardiomyocyte stiffness was determined before and after PKA- or PKC-incubation. To determine whether increased Ca^{2+}-sensitivity was caused by changes in cardiac troponin I (cTnI) phosphorylation, we performed western blot analyses to quantify the amount of unphosphorylated TnI (22B11) and phosphorylation at PKA sites (Ser23/24). Finally we assessed cTnI phosphorylation status by phostag analyses.

Results Titin isoform composition was unchanged. Reduced phosphorylation was observed in titin Ser469 (Fig. A) and Ser170 in PAH compared to donor, while no alteration was observed in Ser26 phosphorylation.

PAH cardiomyocyte passive stiffness was partially restored by PKA treatment (Fig. B), while PKC treatment further increased stiffness. Total unphosphorylated cTnI was significantly higher in PAH and Ser23/24 phosphorylation was lower compared to donor. Phostag analyses confirmed lower cTnI-phosphorylation in PAH compared to non-failing RV (Fig. C).

Conclusions High RV cardiomyocyte Ca^{2+}-sensitivity and passive stiffness are associated with decreased phosphorylation of cTnI and titin in PAH compared to donor. Decreased sarcomere phosphorylation could partially be caused by perturbations in the β-adrenergic receptor/PKA signaling pathway.

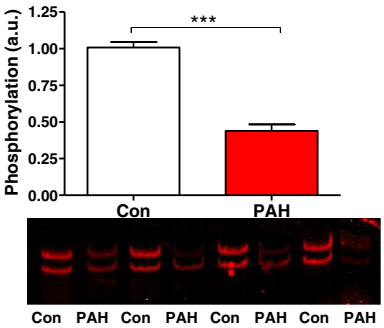

Fig. A Titin N2B domain PKA phosphorylation

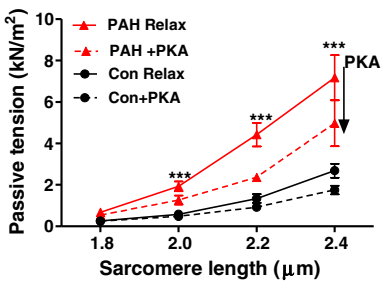

Fig. B PKA effect on PAH and control RV cardiomyocytes

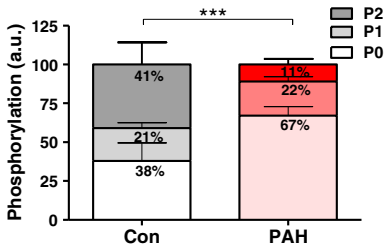

Fig. C TnI phosphorylation

X.P15.

Myofilament changes in doxorubicin-induced dilated cardiomyopathy

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Introduction Several studies demonstrate that administration of doxorubicin, an anthracycline antibiotic used in cancer treatment, results in dilated cardiomyopathy. The present work aims to evaluate the underlying myofilament changes in an animal model of dilated cardiomyopathy.

Methods New Zealand white rabbits were treated with doxorubicin (DOXO, 1 mg/Kg, n = 17) or with an equivolumetric dose of saline (C, n = 16), administered intravenously twice weekly for 8 weeks. Echocardiographic evaluation was performed periodically. At the end of the protocol, myocardial samples were collected to evaluate functional properties of isolated skinned cardiomyocytes in terms of myofilaments active force (Factive), passive force (Fpassive) and calcium sensitivity (pCa50). Titin isoform expression and phosphorylation was quantified and sirius-red stained samples were used to quantify myocardial fibrosis.

Results Echocardiography revealed that DOXO rabbits presented left ventricular (LV) dilation and thinner interventricular and posterior walls. Myocardial fibrosis was similar between groups (C: 0.11 ± 0.01, DOXO: 0.13 ± 0.02 %). Regarding myofilament function, Factive was similar among groups (C: 13.2 ± 1.3; DOXO: 13.3 ± 1.7 mN/mm2), however, Fpassive normalized to Factive was significantly increased in DOXO group (C: 0.20 ± 0.02; DOXO: 0.30 ± 0.05). In DOXO, titin overall phosphorylation was decrease, despite the increased expression of N2BA titin isoform (Fig. 1).

Conclusions The increased expression of the compliant N2BA is consistent with the phenotype of dilated cardiomyopathy observed echocardiographically. Titin phosphorylation deficit underlies the increased myocardial stiffness observed in doxorubicin-induced cardiomyopathy.

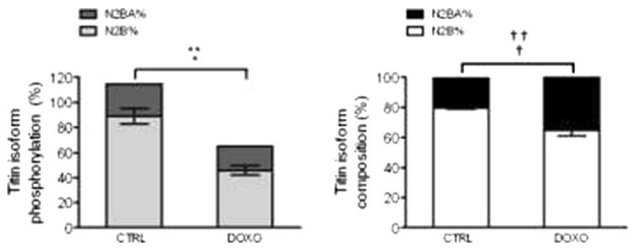

Fig. 1 Titin isoform composition and phosphorylation status

X.P16.

Effect of myosin binding protein-c phosphorylation on ultrastructure of cardiac muscle

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Myosin binding protein-C (MyBP-C) is located within the middle third of each half A-band (C-zone) of the sarcomere and is present along 7–9 stripes of 43 nm periodicity. The cardiac isoform (cMyBP-C) is particularly important as mutations in it are a leading cause of cardiomyopathies. More than one-third of the mutations in MYBPC3 leading to familiar hypertrophic cardiomiopathy are located in the N-terminal domains (C0-C1-m-C2) of the protein. The N-terminal motif region (m domain) is believed to interact with both actin and S2-MHC highlighting the essential role of the phosphorylation of the four serine residues within this domain on cross-bridge kinetics and normal cardiac function.

To study the significance of cMyBP-C phosphorylation in contractile regulation, transgenic mice have been prepared in which the serines are mutated to aspartic acid (constitutive phosphorylation, t3SD) or to alanine (non-phosphorylatable, t3SA). To understand the effect on the fine structure of the sarcomere due to different phosphorylation states we have studied the ultrastructural arrangement of these samples as well as in WT and cMyBP-C-KO. We have carried out electron tomography combined with sub-tomographic averaging to produce mean structures of segments of thick filaments in the C-zone. Results show that, whereas the averaged thick filaments of the WT subject shows good threefold symmetry, both t3SD and t3SA show significant disorder and cMyBP-C-KO lacks the typical threefold symmetry. Here we present our structural findings and discuss their potential roles in the function of the myocardium.

X.P17.

Different vulnerability of subendocardial and subepicardial myocytes to arrhythmia

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We used our mathematical electro-mechanical model of guinea pig cardiomyocyte to study the vulnerability of sub-epicardial (Epi) and sub-endocardial (Endo) myocytes to rhythm disturbances induced by increased density of fast sodium current (I_{Na}) at different modes of contraction. In isometric (heavy-loaded) mode of contractions, increased I_{Na} caused early afterdepolarizations (EAD) in Endo, but not in Epi cells along with rapid fall down of the twitch force and subsequent force recovery at various stimulation frequencies (1, 0.5 Hz). In low-loaded mode of contractions, the increased I_{Na} also caused EAD in Endo cells of the similar patterns as under isometric conditions. In Epi cells, the increased I_{Na} induced delayed spontaneous excitations at 0.5 Hz, which almost stopped contractions. Under the same conditions rhythm disturbances were not observed in Epi cells at 1 Hz. The modeling results suggest that Endo myocytes are more vulnerable to EAD at increased I_{Na} density versus Epi cells. Mechanical conditions of cellular contractions may contribute to arrhythmia induction in Epi myocytes.

X.P18.

A study of a novel phosphorylation site (Ser198) on cardiac troponin I

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Background Ser198 is a novel and unexplored phosphorylation site on cTnI and has significantly altered phosphorylation level in human failing hearts. It is the only phospho-site within the C-terminal fragment proteolyzed in ischemia/reperfusion and cTnI susceptibility to proteolysis is altered when phosphorylated by PKA or PKC.

Objectives To test whether cTnI Ser198 phosphorylation affects myofilament contraction and whether alters cTnI susceptibility to proteolysis.

Methods Human cTnI Ser198 was mutated to Aspartic Acid (S198D) to mimic phosphorylation or Alanine (S198A) to make it non-phosphorylatable. Myofilament functional assays were performed on isolated skinned human idiopathic-dilated-cardiomyopathy myofilaments in which the endogenous troponin complex (Tn) were exchanged with the recombinant Tn containing cTnI variants. Recombinant Tn variants were subjected to Calpain I digestion at 37 °C. The cTnI degradation products were analyzed with immunoblotting.

Results At ~63 % Tn exchange, the myofilaments with S198D showed significantly increased Ca^{2+}-sensitivity of force-development as compared with the ones with S198A and wildtype. At enzyme:substrate = 0.025 unit: 1 ug, immunoblotting with McAb 8I7 detected only one cTnI degradation band and showed significantly increased degradation of mutant cTnI relative to wildtype. However, both MRM mass spectrometry and immunoblotting with cTnI antibodies against its N-terminus, inhibitory region and C-terminus, respectively, showed the major degradation band lost mainly N-terminus not C-terminus.

Conclusions Our results demonstrate that cTnI phosphorylation at Ser198 increases myofilament Ca^{2+}-sensitivity of force development and promotes cTnI susceptibility to proteolysis, indicating phosphorylation of this site could contribute to the altered cardiac function in HF and/or ischemic hearts.

Wednesday September 25 2013

POSTER SESSION XI Muscle Physiology: extreme functional demands

XI.P1

Effects of the intensive care unit condition on craniofacial vs. limb muscle gene and protein expression

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Critical illness myopathy (CIM) is a debilitating and common consequence of modern intensive care, characterized by severe muscle wasting, weakness and a decreased myosin/actin (M/A) ratio. Limb/trunk muscles are primarily affected by this myopathy while craniofacial muscles are spared or less affected. However, the mechanism underlying these muscle specific differences remains unknown. The early drop in M/A ratio in limb muscles in response to the ICU condition is caused by decreased synthesis and increased protein degradation. In this time-resolved study, a unique experimental rat CIM model was used, i.e. a the masseter muscle from immobilized, mechanically ventilated and neuromuscularly blocked rats was studied at durations varying from 6 h to 14 days. Gel electrophoresis, immunoblotting, RT-PCR and morphological staining techniques were used to analyze M/A ratios, synthesis and degradation of myofibrillar proteins and muscle fiber size and compared with previous experimental and clinical results in limb muscles. Significant differences were observed between limb and craniofacial muscles in response to the ICU condition. In contrast to limb muscles, the masseter showed: (1) onlya mild and delayed decline in the M/A ratio, (2) no increase in the atrogenes, (3) no transcriptional downregulation of myofibrillar protein synthesis, and (4) a significant increase in heat shock proteins. However, like limb muscles, the masseter showed an increase in autophagy, activation metalloproteinases and a progressive reduction in fiber size. Consequently, protective mechanisms sustaining myofibrillar protein synthesis and inactivation of the atrogenes are forwarded as important mechanisms underlying the sparing of craniofacial muscles in response to the ICU condition.

XI.P2.

Effects of selenium on the contractile properties of mouse skeletal muscles

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It was shown previously that selenium compounds play a significant role in many physiological functions of the organs, although there is a narrow border between the effective and toxic dose. However the effect of selenium in skeletal muscle have not been studied yet.

In our study we examined the effects of different selenium compounds on muscle properties of mice fed with selenate and NanoSel compounds in different concentrations (0.5, 5, 50 ppm) for 2 weeks.

During our measurements, we measured force in *soleus* and *extensor digitorum longus* (EDL) muscles of the mouse and we detected changes in intracellular Ca^{2+}-concentration on single fibers from *flexor digitorum brevis* (FDB) muscle loaded with fluorescence dye. Both forms of selenium in both muscle types significantly increased the amplitude of single twitches in a concentration dependent manner (in EDL muscle from 99.5 ± 4.7 mN to 127.8 ± 5.1, 140.7 ± 3.6 and 190.8 ± 10.6 mN in case of 0.5, 5 and 50 ppm selenate, respectively). The fatigue of both muscles was reduced by both selenium compounds in the highest concentration during tetanus series (EDL 31 ± 11, 49 ± 5 and 46 ± 1 %; soleus 39 ± 11, 63 ± 5 and 51 ± 6 % control, selenate and NanoSel, respectively). The resting intracellular calcium concentration of the FDB fibers was identical in all group measured. In contrast the amplitude of the calcium transients evoked by KCl depolarization increased significantly from the control 216 ± 22 to 326 ± 60 nM in the presence of 0.5 ppm NanoSel. Our results suggest that selenate and NanoSel improve the contractile properties of skeletal muscles.

XI.P3.

MAO-A inhibition reduces basal oxygen consumption and restores isoprenaline sensitivity of hypertrophied rat papillary muscle in vitro

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Mechanical efficiency (work/oxygen consumption) of RV papillary muscle of pulmonary hypertensive rats is reduced. We hypothesize that the efficiency reduction is due to monoamine oxidase A (MAO-A), which oxidizes catecholamines and produces H_2O_2 on the outer mitochondrial membrane. Thin papillary muscles (volume 0.3–0.6 μl) were isolated from the RV of control and monocrotaline (MCT, 60 mg/kg)-induced pulmonary hypertensive male Wistar rats and mounted in an oxygen chamber equipped with a motor to determine efficiency (work loops at 5 Hz sinusoidal length changes at 37 °C). Mitochondrial characteristics were determined by quantitative enzyme histochemistry and cardiolipin and phosphatydylglycerol by HPLC–MS. The efficiency reduction ($p = 0.027$) and cardiomyocyte hypertrophy ($p = 0.001$) were confirmed. Isoprenaline (1 μM) doubled power output and isometric tension in control papillary muscles. In MCT muscles oxygen consumption doubled ($p = 0.004$) but power output did not increase. MAO-A inhibition by 2 μM clorgyline reduced basal oxygen consumption only in MCT papillary muscles (by 48 SEM 24 μM/s, $p = 0.045$), and restored the inotropic effect of isoprenaline ($p = 0.017$). In the RV free wall of MCT rats the ratio of maximum mitochondrial complex I/complex II activity ($p = 0.032$) and cardiolipin content (CL72:8; $p = 0.023$) decreased, maximum complex II, IV and V activities were similar to control, and phosphatidylglycerol (PG34:1, $p = 0.005$) and MAO-A activity ($p = 0.025$) were increased. Oxidation of catecholamines in hypertrophied cardiomyocytes can prevent positive inotropy and produces a large amount of H_2O_2. MAO-A contributes to the efficiency reduction of hypertrophied myocardium because it consumes oxygen and because the H_2O_2 it produces may cause mitochondrial dysfunction.

XI.P4.

Demonstration of hypoxia in rat papillary muscle using pimonidazole

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Pimonidazole is used as hypoxia marker in tumours and experimentally overloaded hearts. However, the binding constant for oxygen of pimonidazole is unknown in myocardium. We determined the apparent binding constant in isolated rat papillary muscle in vitro. Papillary muscles (n = 3; diameters 0.5–0.7 mm) were dissected from the right ventricle of male Wistar rats. They were mounted in an experimental chamber at optimum length for force production in circulating Tyrode solution at 37 °C. Oxygen tension (PO_{2crit}) at which the preparation developed an anoxic core at 2 Hz stimulation was determined. The muscle was loaded with pimonidazole (2 mg/100 ml; Chemicon International Inc) at high PO_2 without stimulation. Then the preparation was stimulated for 30 min at 2 Hz below PO_{2crit} (360 or 540 mmHg) in Tyrode solution containing pimonidazole. Free pimonidazole was washed away after the stimulation period. Parts of the myocardium served as positive (incubated at $PO_2 = 0$ %, not stimulated) or negative ($PO_2 = 95$ %) control. Pimonidazole binding was demonstrated by immunohistochemistry in cross-sections of the muscles using alkaline phosphatase. Absorbance profiles at 550 nm of pimonidazole binding were determined. The PO_2 profile in the muscle was calculated using a Hill diffusion model. The two profiles were used to construct an exponential relationship between pimonidazole binding and PO_2. The apparent binding constant (0.69/K) was 25 (SEM 3.8) mmHg. We conclude that pimonidazole binding in myocardium can occur at normal oxygen tension. Pimonidazole binding cannot be used to demonstrate myocardial hypoxia in a simple way.

XI.P6.

Observations on 'uremic' muscle: effect of pH on mechanical performance

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The mechanisms behind the weakness and early fatigue of chronic renal disease patients are not fully understood. We aimed to compare the velocity of contraction in permeabilised skinned psoas fibres from healthy and uremic rabbits in normal and low pH.

We induced renal insufficiency (via partial nephrectomy) in New Zealand white female rabbits. Surgery and euthanasia protocols were approved by the University of Thessaly ethics committee. Psoas muscle samples harvested from control (sham-operated) and uremic animals at 3 months were stored in 50 % glycerol solution at -20 °C until mechanical assessments after a 24-h permeabilisation treatment. Basic rigor buffers in mM: 120 KAc, 50 MOPS, 5 $MgAc_2$, 1 EGTA, pH 7, or 50 MES, pH 6.2, (no added phosphate). Relaxing buffer: with addition of 5 mM ATP, 20 mM CP, 1 mg/ml CK. Full activation: with addition of 1.1 mM $CaCl_2$. Experiments using an SI Heidelberg micro dynamometer were performed at 10 °C. Maximal isometric forces were recorded before releases. Data were fit to the Hill equation to estimate maximum velocity of contraction.

Forces and velocities of control fibres (n = 65) were similar to the literature. Uremic muscle fibres (n = 86) however produced significantly lower isometric forces and velocities (67 % of force, 55 % of velocity vs control at pH 7). Lowering of the pH reduced isometric forces to a similar degree for both muscle types (~38 % for control and uremic, each in respect to its pH 7 value, p = 0.000). While velocity was clearly reduced for control (to 77 % of initial value) this was not so much the case for uremic fibres.

Uremic muscle fibres appear to have impaired mechanic performance compared to control even at 'resting' conditions.

Acknowledgments This research has been co-financed by the European Union (European Social Fund—ESF) and Greek national funds through the Operational Program "Educational and Lifelong Learning" of the National Strategic Reference Framework (NSRF)—Research Funding Program: Thales (MuscleFun Project-MIS 377260) Investing in knowledge society through the European Social Fund.

XI.P7

"Mechanical silencing". A primary trigger of critical illness myopathy in intensive care unit patients: experimental and clinical studies and effects of mechanical loadiong

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Critical Illness Myopathy (CIM) is frequently observed in immobilized and mechanically ventilated intensive care unit (ICU) patients. This myopathy is a consequence of modern intensive care and is characterized by paralysis/weakness of limb and trunk muscles, relative sparing of craniofacial muscles, intact sensory and cognitive functions and a preferential myosin loss in affected muscles. However, underlying mechanisms remain poorly understood and CIM is still frequently misdiagnosed as a neuropathy or lumped together with neuropathies, in part due to poor diagnostic techniques/criteria. This potentially lethal complication prolongs the recovery of critical care patients, increases median ICU treatment costs threefold, adds substantial costs for extended rehabilitation requirements and has a significant negative impact on patient quality of life. This study aims at improving understanding of underlying mechanisms by combining clinical with experimental time-resolved analyses using a unique experimental rat ICU model that allows mechanical ventilation with pharmacological post-synaptic paralysis for several weeks. We have examined time-dependent changes in muscle mass and morphology, muscle fractional protein synthesis rate, intracellular signalling, myofibrillar gene and protein expression, as well as regulation of muscle contraction at the single fibre level. Results show that the complete mechanical silencing is a primary factor underlying and the study has therefore been extended to study the cellular and molecular effects of mechanical loading in both experimental and clinical studies.

XI.P8.

Chronic hypoxia induces oxidation of proteins central to many homeostatic processes in mouse diaphragm muscle

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Chronic hypoxia (CH)-induced functional remodelling in the diaphragm may be associated with alterations in redox homeostasis. We assessed protein redox modifications and antioxidant activity over time in the diaphragm of mice exposed to CH. Furthermore, we used a 2D redox proteomics to identify modified proteins after 6 weeks CH exposure.

C57Bl6J mice were exposed to one, three and 6 weeks of CH (10 % F_iO_2) or normoxia. Muscle homogenates were incubated with carbonyl- or thiol-reactive fluorophores before 1D/2D-PAGE and fluorescence scanning. Proteins of interest were identified by mass spectrometry. Catalase activity was determined by following the decomposition of H_2O_2 spectro-photometrically.

Increased carbonylation and decreased thiol-content was observed after 6 weeks of CH ($p < 0.0001$ and $p < 0.01$respectively; $n = 7–8$ per group; Student's *t* test). No change in carbonylation was detected after 1 week of CH, but there was an increase in thiol content ($p < 0.001$). Furthermore, catalase activity in the diaphragm was significantly increased and maintained after one, three and 6 weeks of CH ($p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively) suggesting an early antioxidant response that becomes overwhelmed. Significantly modified proteins identified regulate metabolic switching, Ca^{2+}-handling, iron homeostasis and various signalling pathways. Expression changes were observed to stress-response and chaperone proteins such as 60 kDa HSP ($p < 0.01$) and α-Crystallin-B-chain ($p < 0.001$).

An early endogenous antioxidant response cannot prevent accumulation of protein oxidation in hypoxic mouse diaphragm. Modified proteins suggest the mitochondria are the source of oxidants but the stress reaches other organelles and the cross-bridge.

XI.P9.

Observations on the effect of uremia on isometric force

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Chronic renal patients present with muscle atrophy and muscle weakness. The muscle dysfunction leads to decreased mobility, low quality of life and premature death in this clinical population.

We aimed to evaluate the difference in maximal isometric force of permeabilised skinned psoas fibres from healthy and uremic rabbits at various temperatures.

We induced renal insufficiency (via partial nephrectomy) in New Zealand white female rabbits. Surgery and euthanasia protocols were approved by the University of Thessaly ethics committee. Psoas muscle samples harvested from control (sham-operated) and uremic animals at 3 months were stored in 50 % glycerol solution at −20 °C until mechanical assessments after a 24-h permeabilisation treatment. Maximal isometric tension (Po) ($n = 152$, 86 uremic/66 control) was assessed in 3 temperatures (10, 15, 30 °C) using an SI Heidelberg micro dynamometer. Basic rigor buffer in mM: 120 KAc, 50 MOPS, 5 $MgAc_2$, 1 EGTA, pH 7. Relaxing buffer: with addition of 5 mM ATP Full activation: with addition of 1.1 mM $CaCl_2$. A general linear model (GLM) for repeated measures was used to compare force over the 3 temperatures between the two groups. If the global tests from GLM were significant, Bonferroni's tests were used for pairwise comparisons.

Uremic muscle fibres produced significantly lower isometric forces in all three temperatures studied compared to sham operated control fibres (10C: 67 %, 15C: 63 %, 30C: 67 %, $p = 0.000$). There was no significant interaction between temperature and condition.

Uremic muscle fibres produced consistently lower maximal isometric forces whether at low or high temperatures at maximal calcium activation at resting conditions. Work is underway to tease out the mechanism responsible for the lower force production.

Acknowledgments This research has been co-financed by the European Union (European Social Fund—ESF) and Greek national funds through the Operational Program "Educational and Lifelong Learning" of the National Strategic Reference Framework (NSRF)—Research Funding Program: Thales (MuscleFun Project-MIS 377260) Investing in knowledge society through the European Social Fund.

XI.P10.

Pharmacological ampk activation improves skeletal muscle mitochondrial function and muscle endurance in aged myostatin KO mice

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Myostatin (mstn) blockade, resulting in muscle hypertrophy, is one of the most promising therapies to counteract age-related muscle loss. However, mitochondrial defects, oxidative metabolism and muscle function alteration are observed in young mstn KO mice, and could be detrimental with aging. This raised the interest of activating mitochondrial biogenesis and metabolism to improve aged mstn-deficient muscle function. Chronic AMPK activation is one way to induce mitochondrial biogenesis and up-regulate oxidative metabolism. The aim of study was to investigate in aged mstn KO and wild-type (WT) mice the muscle function and metabolism response to 4-weeks AMPK-activating treatment, using AICAR (5-aminoimidazole-4-carboxamide-1-D-ribofuranoside). Data showed, in glycolytic muscles, that the hypermuscular phenotype, associated to a reduced aerobic metabolism (decreased PGC-1α, VDAC protein expression, citrate synthase activity, fatty acids transporters expression, increased glucose transporter expression; $p < 0.05$) and decreased running performance, was persistent in aged mstn KO mice. AICAR treatment increased PGC-1α protein expression, a downstream target of AMPK, in WT but markedly in mstn KO mice ($p < 0.05$). No significant impact of AICAR treatment on mitochondrial content in both groups was observed. AICAR treatment improved respiratory complexes activities, and running time only in aged mstn KO mice showing differential genotype AICAR effect. Collectively, our results underscore the mstn blockade, as effective strategy to counteract age-related muscle loss, but underline in parallel the importance to improve skeletal muscle oxidative quality in aged mstn KO mice. Interestingly, this study showed that impaired muscle function in aged myostatin-deficient mice can be improved by AICAR, an exercise mimetic drug.

XI.P11.

A comparison of inflammatory and functional effects of different cold-water immersion protocols for recovery from high-intensity sprint exercise

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Cold-water immersion and other forms of cold therapy are commonly used following exercise to reduce delayed onset muscle soreness and speed the return of muscle performance. It is thought that the therapeutic and/or recovery effects of cold to this end is due to its anti-inflammatory action. Although it is commonly used by athletes of all types and by exercise and rehabilitation scientists alike, no protocol (duration of immersion/application or temperature) has been identified nor has the anti-inflammatory mechanistic theory been validated. We are conducting a comparison of four different cold-water immersion protocols of different duration and temperature for their efficacy to reduce plasma markers of inflammation and speed recovery of decreased performance induced by high-intensity sprint exercise. In a randomized cross-over study, we compared four different cold-water immersion protocols and passive recovery for their efficacy to reduce plasma markers of inflammation and speed recovery of decreased performance induced by high-intensity sprint exercise. $n = 8$ recreationally active male subjects performed 12×120 m sprints on an indoor track. Immediately following exercise, subjects were randomly assigned to one of four cold-water immersion protocols (10° for 10 min, 20° for 10 min, 10° for 30 min, 20° for 30 min) or passive seated rest. Performance on squat jumps (SJ) and drop jumps (DJ), subjective measures of soreness and perceived recovery, and plasma levels of pro-inflammatory markers (MPO, GM-CSF, IFN-y, IL-1B, IL2, IL-6, IL-8, IL-10, IL-12p70, and TNF-a) were measure at time-points pre-exercise, post-exercise, 1 h, 2 h, 24 h, and 48 h post-exercise. Results have not yet been analyzed and assays for plasma pro-inflammatory markers are also currently being analyzed. We expect that measures for soreness will positively correlate with pro-inflammatory markers and DJ performance, SJ performance, and ratings of perceived recovery will negatively correlate with pro-inflammatory markers.

Conclusions Pseudo-phosphorylation of Ser42/44 decreases Ca^{2+}-sensitivity and blunts length-dependent activation in response to PKA in human cardiomyocytes. Since the drop in Ca^{2+}-sensitivity at physiological [Ca^{2+}] is relatively large, our measurements suggest that PKC-mediated phosphorylation of Ser42/44 results in a decrease of force and associated ATP utilisation in the human heart.

POSTER SESSION XII Mitochondrial function in health and disease

XII.P1

Contractile properties of skeletal muscle myofibrils isolated from mice with impaired activity of the enzyme arginyl transferase (ATE1)

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Introduction Protein arginylation is a post-translational process catalyzed by arginyl-tRNA-protein transferase (Ate1), an enzyme that transfers arginine from tRNA onto proteins. Recent data from our laboratory suggests that arginylation represents a major regulator of muscle contractility. The purpose of this study was to investigate the role of arginylation on contractile properties of skeletal muscle myofibrils.

Methods Myofibrils were isolated from the soleus muscle taken from wild type (WT) mice and knockout mice lacking the enzyme Ate1 (Ate1 KO). The samples were homogenized, and individual myofibrils were attached between an atomic force cantilever and a rigid glass micro-needle. Active force, passive force and the rates of force development (K_{act}), redevelopment (K_{tr}) and fast phase of relaxation (K_{rel}) were measured in sarcomeres lengths (SL) ranging between 2.2 and 3.0 μm. The content of myosin heavy chains (MHC) was measured to identify potential shifts in muscle types.

Results Myofibril from Ate1 KO showed a reduction of active force (WT 190 ± 22mN mm^{-2}; Ate1 KO 162 ± 18mN mm^{-2}) and passive forces. The K_{act} (WT 1.8 ± 0.4 s^{-1}; Ate1 KO 1.3 ± 0.3 s^{-1}), K_{tr} (WT 2.2 ± 0.3 s^{-1}; Ate1 KO 1.7 ± 0.4 s^{-1}) and K_{rel} (WT 2.0 ± 0.4 s^{-1}; Ate1 KO 1.9 ± 0.3 s^{-1}) were slower in Ate1 KO than in WT myofibrils. The ratio between MHC I and MHC IIa was not changed in the Ate1 KO group.

Conclusion Arginylation is an important regulator of skeletal muscle contractility, affecting the kinetics of myosin crossbridges and altering force production and muscle contraction.

XII.P3.

Observations on the effects of uremia on redox status

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In the later stages of renal disease, patients present with muscle weakness, metabolic disorders, skeletal muscle atrophy and fatigue. The exact mechanisms of muscle dysfunction are not yet understood, however oxidative stress may be implicated.

The aim of the study was to evaluate the effects of uremia on redox status in blood and muscle tissue homogenates from healthy and uremic rabbits.

We are using an animal model (partial nephrectomy) of renal disease in New Zealand white female rabbits. Surgery and euthanasia (after 3 months) protocols are approved by the ethic committee of the University of Thessaly. Blood samples were collected and separated in serum, plasma and red cell lysate. Muscle tissue samples from psoas muscle were harvested and homogenized. Samples were stored at −20 °C until analyzed for catalase activity, total antioxidant capacity and uric acid in spectrophotometry and biochemical analyzer.

Analysis has been done so far in 3 rabbits [1 control (sham operated)/ 2 uremic (partial nephrectomy)]. Nephrectomy resulted in deteriorated biochemical profile (marked increases in urea, creatinine). Uremic rabbits showed a 40 % reduction in catalase activity, 24 % reduction in total antioxidant capacity and 62 % reduction in uric acid concentration in blood. Similar results were also noticed in psoas muscle tissue homogenates.

The evaluation of clinical indices, urea and creatinine, imply an advanced renal failure in the nephrectomized rabbits. A reduction in antioxidant capacity of uremic rabbits in comparison with control was observed. Work is underway to clarify the possible contribution of oxidative stress on muscle functionality.

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XII.P4.

The impact of oxidative stress upon physiological parameters and proton transverse relaxtion times in aging rat heart

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Ischemia is a frequent phenomenon associated with aging, which induces changes in the distribution and polarization of tissue water which in turn may influence proton transverse relaxation times. The aim of this study was to point out changes produced by the oxidative stress represented by 30 min ischemia followed by 60 min reperfusion of isolated rat hearts of different ages upon physiological parameters of cardiac contractility such as: heart rate (H.R.), coronary flow (C.F.) and left ventricle systolic pressure (L.V.S.P. as well as to evaluate proton transverse relaxation times in fresh and glycerinated cardiac muscle incubated with Rigor and relaxation solutions. Isolated rat hearts of 6 and 37 months old have been mounted and perfused with Krebs Hanseleit buffer at 37 °C in Langendorff retrograde perfusion system at a constant pressure over 60 min (i.e. at 10′,20′,30′,40′,50′,60′) intervals have been determined: H.R., C.F. and L.V.S.P. Our data have pointed out that, in old rats H.R. exhibits higher values than in young controls.C.F. is variable in time in aging rats versus young ones where is a slow decrease during the experiment. LVSP is net elevated in aging rats, but with fluctuations in time. T2s in rigor is shorter than in relaxation and these differences are more obvious in glycerinated muscle. These values of T2s may offer informations about oxidative insults of myofilaments even when the functional parameters of heart are not modified yet. The ratio between T2s in e Ri and Re media may be a valuable parameter which indicates without any doubt a significant modification coused by oxidative stress at the level of contractile apparatus.

XII.P5.

Role of oxidative stress induced by monoamine oxidases in myoblasts of patients with collagen VI myopathies

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Muscular dystrophies (MDs) are genetic, progressive diseases for which no routine effective therapies are yet available. In Ullrich Congenital Muscular Dystrophy (UCMD) and Bethlem Myopathy (BM), caused by abnormalities of Collagen VI, muscle fibers degenerate and die. The absence of Collagen VI has been causally related to mitochondrial dysfunction. Although several studies documented the key role of oxidative stress and increased formation of reactive oxygen species (ROS) along with mitochondrial dysfunction in the pathophysiology of these diseases, the source of ROS remains ill-defined.

Based upon our previous results in murine models of muscular dystrophy (Menazza et al. 2010, *Hum. Mol. Genet.* 19, pp. 4207–4215), we investigated whether the mitochondrial enzymes monoamine oxidases (MAOs) contribute to oxidative stress and mitochondrial dysfunction in myoblasts from four patients affected by UCMD and one patient affected by BM.

MAO protein levels were higher in myoblasts from patients with collagen VI myopathies. Accordingly, when myoblasts were incubated with the MAO substrate tyramine, ROS formation was higher in cells from patients as compared to healthy donors. Increased ROS formation was significantly reduced by MAO inhibition with pargyline or safinamide. Notably, the decrease in ROS level was paralleled by improved mitochondrial function and maintenance cell viability.

Taken together, these findings confirm the relevance of MAO-dependent ROS formation in collagen VI myopathies, thus providing the rationale for future clinical trials by using compounds, such as MAO inhibitors, that are currently used for neurological disorders.