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EUROMIT 6

Foreword

Dear Participants,

On behalf of the local Organizing Committee of the University Medical Centre Nijmegen, it is my pleasure to welcome you to the sixth European Meeting on Mitochondrial Pathology (Euromit 6).

In the year 2004, the University Children's Hospital, to which the Nijmegen Centre for Mitochondrial Disorders, the principle organisers of this meeting, belongs, celebrates its fiftieth anniversary with lots of different activities, the Euromit 6 meeting being one of the most important scientific ones.

The number of participants of the Euromit meetings is steadily growing. This year we will have around 400 participants from all over the world! The success of the Euromit meetings can be explained not only by the high scientific level covering the whole spectrum from basic science to clinical research but is also due to the increased importance mitochondrial energy metabolism has in a broad range of different disease areas.

In this abstract book, you will find all the contributions of the participants of Euromit 6. The programme with invited speakers, free communications and a timetable is included. Also, as a result of this conference, a special BBA issue—sponsored by Elsevier—will be published shortly after the meeting. This special issue will contain articles from the invited speakers.

The conference will take place in the ''Studiecentrum der Medische Wetenschappen'' of the University Medical Centre Nijmegen. Lectures will be held in the ''Lammers Zaal'' on the first floor. A slide-preview room, a computer room with access to e-mail and the Internet, and a room for small meetings (approximately 25 people maximum) are available at the ''Studiecentrum''.

I wish you a most exciting and enjoyable meeting, as well as a great time in Nijmegen.

Prof. Dr. Jan A.M. Smeitink
Chairman Nijmegen Centre for Mitochondrial Disorders

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- Prof. Dr. Jirri Zeman
- Prof. Dr. Massimo Zeviani

Daily Programme Euromit 6

Thursday 1st July 2004

09:00–09:05	Welcome *Jan Smeitink/Rob Sengers*
09:05–09:30	**Opening lecture: Mitochondrial medicine** *Chairperson Rob Sengers* Salvatore DiMauro
	Clinical presentation and disease course *Chairperson Jirri Zeman*
09:30–10:00	Clinical presentation and disease course of mitochondrial disease in children *Arnold Munnich*
10:00–10:30	Clinical presentation and disease course of mitochondrial disease in adults *Douglass Turnbull*
	Free communications
10:30–10:45	Surprising phenotypes of mtDNA maintenance protein defects *Anu Suomalainen-Wartiovaara*
10:45–11:15	**Coffee break**
	Epidemiology and noninvasive techniques *Chairperson Kari Majamaa*
11:15–11:45	The epidemiology of mitochondrial disorders *Patrick Chinnery*
11:45–12:15	Magnetic resonance in mitochondrial disorders *Marjo van der Knaap*
	Free communications
12:15–12:30	Clinical spectrum, morbidity, and mortality in 113 patients with mitochondrial disease *Fernando Scaglia*
12:30–12:45	Brain MRI and proton MR spectroscopy (MRS) in the diagnostic evaluation of children with respiratory chain (RC) defects *Argirios Dinopoulos*
12:45–14:00	**Lunch**
	Biochemical and molecular diagnostics: new developments *Chairperson Frans Trijbels*
14:00–14:30	Biochemical and molecular diagnostics: how do we decide what gene(s) to investigate? *David Thorburn*

14:30–15:00	Molecular diagnostics: new developments *Agnès Rötig*
	Free communications
15:00–15:15	Consensus protocols for the spectrophotometric assays of respiratory chain activities *Anne Lombes*
15:15–15:30	Automated analysis of respiratory chain complex (RCC) enzyme activities in cultured skin fibroblasts *Sihoun Hahn*
15:30–16:00	**Coffee/tea**
	Gene hunting strategies and new genetic defects *Chairperson Bert van den Heuvel*
16:00–16:30	New genes in mitochondrial disorders *Massimo Zeviani*
16:30–17:00	How to find genes for recessive oxidative phosphorylation disorders, and what to do once you have found them *Eric Shoubridge*
	Free communications
17:00–17:15	Truncated product of the bifunctional DLST gene involved in the assembly of the mitochondrial respiratory complexes and associated with Alzheimer's disease *Shigeo Ohta*
17:15–17:30	Pyruvate dehydrogenase E1 beta subunit deficiency *Garry Brown*
17:30–18:30	**Posters and bar**
18:30–20:00	**Dinner**
	Evening lecture *Chairperson Jan Smeitink*
20:00–21:00	Structural proteomics of mitochondria *Sir John Walker*

Friday 2nd July 2004

	mtDNA replication, maintenance and transcription *Chairperson Palmiro Cantatore*
09:00–09:30	Replication of mitochondrial DNA *Ian Holt*
09:30–10:00	Mammalian mitochondrial transcription and DNA replication: in vitro reconstitution and molecular characterization *Claes Gustafsson*
	Free communications
10:00–10:15	The majority of TFAM molecules are required for maintenance of mitochondrial DNA and a minority for transcription: TFAM-titration model *Dongchon Kang*
10:15–10:30	Organization and dynamics of human mitochondrial DNA *Manuel Rojo*
10:30–11:00	**Coffee break**
	Import and protein degradation *Chairperson Sergio Papa*
11:00–11:30	The chaperone/Tom70 mitochondrial targeting pathway *Jason Young*
11:30–12:00	Regulation of mitochondrial activity by proteolysis *Thomas Langer*
	Free communications
12:00–12:15	Chaperone-like activities of a new cytosolic factor AIP and import receptors Tom20 and Tom22 facilitate mitochondrial protein import *Masataka Mori*
12:15–12:30	Biogenesis of the mitochondrial inner membrane *Carla Koehler*

12:30–14:00	**Lunch**
	Biogenesis and assembly
	Chairperson Leo Nijtmans
14:00–14:30	Mouse models of isolated cytochrome oxidase deficiency *Carlos Moraes*
14:30–15:00	Molecular genetics of complex I-deficient Chinese hamster cell lines *Immo Scheffler*
	Free communications
15:00–15:15	Alternative topogenesis of Mgm1 is essential for the integrity of mitochondrial structure *Mark Herlan*
15:15–15:30	COX assembly: Mss51p and Cox14p jointly regulate Cox1p expression in the yeast *S. cerevisiae* *Antoni Barrientos*
15:30–16:00	**Coffee/tea**
	Cell biological consequences of OXPHOS disease
	Chairperson Bé Wieringa
16:00–16:30	Respiratory chain defects: what do we know for sure about their consequences in vivo? *Pierre Rustin*
16:30–17:00	Dying mitochondria in agonizing cells *Guido Kroemer*
	Free communications
17:00–17:15	Mutation-specific mitochondrial remodeling in complex I-deficient patient fibroblasts *Werner Koopman*
17:15–17:30	Miro, a novel GTPase with Ca^{2+}-binding EF-hand motifs, may integrate cell-signaling pathways with mitochondrial dynamics *Koji Okamoto*
17:30–18:30	**Posters and bar**
18:30	**Free evening**

Saturday 3rd July 2004

	Models
	Chairperson Nils Larsson
09:00–09:20	Mitochondrial disease in flies *Howy Jacobs*
	Free communications
09:20–09:30	Micro-arrays and TFAM knock-out hearts: a switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts *Anna Hansson*
09:30–09:45	Modelling mitochondrial complex I disorders in the nematode *Caenorhabditis elegans* *Leslie Grad*
	RNA profiling-Chip technology
	Chairperson Massimo Zeviani
09:45–10:15	RNA profiling-Chip technology *Douglas Wallace*
	Free communications
10:15–10:30	Gene expression profiles define mitochondrial pathology in yeast mutants and human disease *Hubert Smeets*
10:30–11:00	**Coffee break**
	Mitochondrial proteomics
	Chairperson John Walker
11:00–11:30	Immunological and proteomics approaches to characterisation of mitochondrial diseases *Rod Capaldi*
	Free communications
11:30–11:45	Integrative analysis of the mitochondrial proteome *Holger Prokisch*

	Mitochondria and cancer
	Chairperson Patrick Chinnery
11:45–12:15	Mitochondria and cancer
	Lauri Aaltonen
	Free communications
12:15–12:30	Succinate dehydrogenase and mitochondrial signalling in HIF1a stabilization
	Mary Selak
12:30–14:00	**Lunch**
	Free afternoon
19:30	**Conference dinner**

Sunday 4th July 2004

	Bio-informatics
	Chairperson Eric Shoubridge
09:00–09:25	Reconstructing proto-mitochondrial metabolism
	Martijn Huynen
	Models
	Chairperson Eric Shoubridge
09:25–09:50	*Yarrowia lipolytica*, a model organism to study mitochondrial complex I by yeast genetics
	Ulrich Brandt
09:50–10:15	Creation of mtDNA mutator mice
	Aleksandra Trifunovic
	Free communications
10:15–10:30	Mitochondria-mediated nuclear mutator phenotype in *Saccharomyces cerevisiae*
	Keshav Singh
10:30–11:00	**Coffee break**
	Treatment strategies
	Chairperson Laurence Bindoff
11:00–11:25	Treatment strategies for disorders of the mitochondrial genome
	Bob Lightowlers
11:25–11:50	Use it or lose it?—Exercise training implications of mtDNA disorders
	Tanja Taivassalo
	Free communications
11:50–12:05	Aerobic training improves oxidative capacity in patients with mitochondrial myopathy
	John Vissing
12:05–12:20	Ketogenic treatment reduces the proportion of mutated mitochondrial DNAs
	Eric Schon
12:20–12:40	**Surprise box**
	Chairperson Douglass Turnbull
12:40–12:45	Synopsis
	Douglass Turnbull
12:45	**End of programme**

Invited and free communications according to programme

I-1
Mitochondrial medicine

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The concept of mitochondrial disease was introduced in 1962, when Luft and coworkers described a woman with severe hypermetabolism not due to ''loose coupling'' of oxidative phosphorylation in muscle mitochondria. During the decade that followed, the attention of clinical scientists was largely directed to muscle morphology: ''megaconial'' and ''pleoconial'' myopathies were described by Shy and Gonatas, and the ''ragged-red fibers'' (RRF) were introduced by W. King Engel.

In the 1970s, biochemical studies began identifying specific metabolic defects, leading to a classification of the mitochondrial diseases into five major groups: (i) defects of substrate transport; (ii) defects of substrate utilization; (iii) defects of the Krebs cycle; (iv) defects of the electron-transport chain; and (v) defects of oxidation/phosphorylation coupling. However, the term mitochondrial diseases has been restricted to defects of the respiratory chain. This conventional wisdom is supported by the biochemical complexity of the respiratory chain and by its dual genetic control, explaining the extraordinary clinical and genetic heterogeneity of mitochondrial diseases.

The ''molecular age'' started in 1988, when mitochondrial DNA (mtDNA) deletions were associated to myopathy by the late Anita Harding and her coworkers in London, and a point mutation in the mtDNA ND4 gene was associated to Leber's hereditary optic neuropathy (LHON) by Doug Wallace's group in Atlanta.

I-2
Clinical presentation and disease course of mitochondrial disease in children

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Owing to the ubiquitous nature of oxidative phosphorylation, a defect of the mitochondrial respiratory chain should be considered in any unexplained association of symptoms, with a progressive course and involving seemingly unrelated organs or tissue. The disease may begin at virtually any age, even antenatally. Reviewing a large series of proven respiratory chain deficiency, we investigated the antenatal manifestations of oxidative phosphorylation disorders in our series. A total of 300 cases of proven respiratory enzyme deficiency were retrospectively reviewed for fetal development based on course and duration of pregnancy, antenatal ultrasonography and birth weight, length and head circumference. Particular attention was given to fetal movements, oligo/hydramnios, fetal cardiac rhythms and heart ultrasounds (US) and US/echodoppler signs of brain, facial, trunk, limb and organ anomalies. Retrospective analyses detected low birth weight (below the 3rd centile for gestational age) in 23% of cases (68/300, $P = 0.000001$). Failure to thrive was either isolated (48/300, 16%) or associated with otherwise unexplained anomalies (20/300, 7%, $P = 0.0001$). Antenatal anomalies were usually multiple and involved several organs sharing no common function or embryological origin. The following anomalies were observed: polyhydramnios (6/20), oligoamnios (2/20), arthrogryposis (1/20), decreased fetal movements (1/20), ventricular septal defects (2/20), hypertrophic cardiomyopathy (4/20), cardiac rhythm anomalies (4/20), hydronephrosis (3/20), VACTERL association (2/20) or a complex gastro-intestinal malformation (1/20). Central nervous system anomalies included cerebellar agenesis, porencephalic cysts and enlarged ventricles. These anomalies remained unexplained during pregnancy and only ascribed to the disease postnatally. They were not related to the type or severity of enzyme deficiency.

While a number of metabolic diseases undergo a symptom-free period, it appears that respiratory chain deficiency may have an early antenatal expression, probably related to the hitherto unknown time course of disease gene expression in the embryofetal period.

I-3
Clinical presentation and disease course of mitochondrial disease in adults

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The clinical features of mitochondrial disease in adults are extremely variable. Some patients present with symptoms and signs which are well recognised in mitochondrial disease such as chronic progressive external ophthalmoplegia or diabetes and deafness. However, many patients do not present with phenotypes that are readily recognised as mitochondrial disease and making a clinical diagnosis remains a considerable challenge. The involvement of different systems or the presence of a maternally family history may be an important clue to the diagnosis in some patients. Many adult patients with mitochondrial disease have defects of the mitochondrial

genome and some of the clinical variability relates to the degree of heteroplasmy observed in different patients.

Our group has been collecting follow-up data from our patients with mitochondrial disease for several years and for some patients the disease has a benign clinical course. However, for others close follow-up is essential to monitor the development of treatable clinical problems such as diabetes and cardiac conduction defects. Collecting information on the natural history of specific mitochondrial diseases is important to develop guidelines for management for individual patients.

O-1
Surprising phenotypes of mtDNA maintenance protein defects

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Mutations of mitochondrial replication helicase Twinkle and mitochondrial DNA polymerase gamma (POLG) have recently been shown to underlie inherited progressive external ophthalmoplegia (PEO) and result in accumulation of multiple mtDNA deletions in the patients' tissues. Typical findings are COX-negative fibres in muscle biopsy sample and multiple mtDNA mutations in Southern blot.

We have characterized in detail the clinical and molecular genetic features of our family material with a variety of neurological disturbances, for mutations in Twinkle and POLG. We focused on such families, who had some symptoms described in PEO-families, such as Parkinsonism, polyneuropathy and ataxia. All these families did not have PEO.

We describe here two completely new neurodegenerative phenotypes with POLG or Twinkle mutations, not previously associated with mtDNA maintenance proteins. These patients do not have PEO. These patients have been completely missed in routine DNA and morphological diagnostic procedures, since they do not show morphological changes in the muscle suggestive of mitochondrial myopathy, nor do they have mtDNA deletions detectable by Southern blot analysis. We have identified a founder mutation in Finland for an adult-onset recessive neurodegenerative disease, the prevalence of which exceeds Friedreich's ataxia in Finland. We also describe an infantile neurodegenerative disorder associated with recessive mutations of Twinkle. This is the first report of recessive Twinkle mutations. Thirdly, we also report highly significant ($P<10^{-6}$) co-segregation of Parkinsonism in our PEO families with dominant POLG mutations.

Our results show that mtDNA maintenance protein defects can cause CNS-restricted neurodegenerative phenotypes of infantile and adult onset.

I-4
The epidemiology of mitochondrial disorders

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Until recently mitochondrial disorders were thought to be extremely rare, only affecting one or two per million of the population, but recent epidemiological studies in Europe and Australia have shown that, as a group, mitochondrial diseases are amongst the most common inherited disorders, affecting at least 1 in 10,000 of the general population. The story is, however, far from complete. With the increasing awareness of mitochondrial disease amongst non-specialist physicians, greater availability of biochemical and molecular tests, and an ever expanding phenotype, the true prevalence of mitochondrial disease is likely to be much greater than the published figures. Over the last 5 years, we have continued to identify new cases within a relatively small, well-defined area. Detailed family studies have revealed an increasing number of oligosymptomatic cases in families with mtDNA and nuclear genetic disorders. A number of additional questions also remain unresolved. Does the incidence vary in different ethnic groups, and what are the genetic and environmental factors that influence the penetrance of different mtDNA mutations? These issues have important implications for our understanding of the pathogenesis of mitochondrial disease, and the provision of health care to patients and their families.

I-5
Magnetic resonance in mitochondrial disorders

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In mitochondrial disorders, magnetic resonance imaging (MRI) and spectroscopy (MRS) are important mainly in two aspects: diagnosis and monitoring.

MRI of the brain may facilitate a diagnosis in patients who present with an encephalopathy. Different patterns of abnormalities can be recognized:

a pattern of energy failure, consisting of cortical pseudoinfarcts, basal ganglia lesions and brain stem lesions, in the acute phase often with contrast enhancement.

a pattern of chronic neurodegeneration with global cerebral and/or cerebellar atrophy.
a pattern of focal or diffuse white matter abnormalities, often with cystic degeneration and areas of contrast enhancement.
malformations are rare and may include agenesis of the corpus callosum and cortical dysplasia.

The first pattern is typically seen in MELAS, Leigh syndrome and NARP. The second pattern is seen in MERFF. The third pattern may be seen in complex I deficiency, succinate dehydrogenase deficiency, cytochrome *c* oxidase deficiency and MNGIE. The fourth pattern is seen in severe variants of pyruvate dehydrogenase complex deficiency. Patients often display a combination of abnormalities from different patterns on their MRIs. Cerebral white matter abnormalities in combination with lesions in the basal ganglia and brain stem are relatively frequent and, for example, typical for Kearns–Sayre syndrome. Many patients with significant cerebral white matter abnormalities on the basis of a mitochondrial disorder have basal ganglia lesions. The latter feature facilitates the diagnosis.

Characteristic for mitochondrial disorders is that abnormalities may come and go and be entirely or partially reversible. This makes MRI useful in the monitoring of abnormalities over time and under treatment.

Proton MRS reveals variable elevations of lactate in mitochondrial disorders, although lactate is not elevated in all patients. Some found a good correlation between brain and CSF levels of lactate, but discrepancies have been described repeatedly. There is a regional variability in elevations of cerebral lactate, lactate elevations being most pronounced in regions where MRI shows fresh structural abnormalities. Lactate decreases again when the lesion turns into inactive scar tissue. In pyruvate dehydrogenase complex deficiency, elevated cerebral pyruvate may be demonstrable in addition to the elevated lactate. In patients with succinate dehydrogenase deficiency, elevated succinate can easily be detected. In addition, spectroscopic changes are present related to tissue damage, including a decrease in *N*-acetylaspartate, glutamate and creatine, related to neuronal dysfunction and loss. The spectroscopic features can be used to monitor the course of disease, in particular to evaluate effects of treatment.

O-2
Clinical spectrum, morbidity, and mortality in 113 patients with mitochondrial disease

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Mitochondrial disorders are clinical entities associated with abnormalities of oxidative phosphorylation. Since their natural history remains largely unknown, the aim of this study was to investigate the clinical history of pediatric mitochondrial disorders based on different presenting features. By using the modified Walker criteria, 113 patients with definite mitochondrial disease were identified. Respiratory chain (RC) defects were found in 71% of the patients who underwent a muscle biopsy. Complex I (32%), and combined complex I, III, and IV deficiencies (26%) were the most common causes of RC defects, followed by complex III (19%), complex IV (16%), and complex II deficiencies (7%). MtDNA abnormalities were found in 11% of the patients. Forty-two percent of the children studied exhibited cardiomyopathy, however, the majority (58%) had predominant neuromuscular manifestations. No correlation between the type of RC defect and the clinical presentation was noted. The mean age of diagnosis was 33 months in the cardiac group and 44 months in the non-cardiac group ($P < 0.005$). Patients with cardiomyopathy had an 18% survival rate at 16 years of age compared to patients with neuromuscular features, who had a 90% survival at the same age ($P < 0.0001$). This study supports the concept that in patients with RC defects, cardiomyopathy is more common than previously believed, and that patients with cardiomyopathy follow a more severe clinical course, regardless of the associated RC defect. MtDNA mutations were found in a minority of patients, underlining the fact that most of the mitochondrial disorders of childhood follow a Mendelian pattern of inheritance.

O-3
Brain MRI and proton MR spectroscopy (MRS) in the diagnostic evaluation of children with respiratory chain (RC) defects

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Objective: To compare the morphological and regional metabolite changes with MRI and MRS in patients with RC defects.

Method/Results: We retrospectively evaluated MRI and MRS studies in 47 children (mean age of presentation 1.7 years) with RC deficiency. Patients were divided in three groups (22 ''definite'', 14 ''probable'', 11 ''possible'') according to Modified Adult Criteria for the diagnosis of RC defect (lactate peak on MRS was not used as criterion). Imaging abnormalities were compared in three groups using x^2 statistics.

83 MRI and 55 MRS were reviewed (mean age of first imaging 3.5 years). All patients with "pure" myopathy had normal initial and follow-up studies. Abnormalities in patients with encephalomyopathy were in "definite"/"probable"/"possible", respectively:

Deep-gray matter: 42%/7.7%/0% $\{\times^2 = 8.1$ df = 2 $P = 0.02\}$
Delay of myelination: 32%/46%/37%
Leukoencephalopathy: 26%(subcortical involvement)/31%/25%
Cerebral atrophy: 37%/23%/12%
Cerebellar atrophy: 47%/38%/0%
Lactate peak: 79%/36%/0% $\{\times^2 = 13.2$ df = 2 $P = 0.01\}$
Low NAA/Cr: 64%/27%/25%

Conclusion: The most prominent structural abnormality, mainly in patients with definite RC defect, is the deep gray-matter involvement and represents a useful diagnostic marker for the disease. Delay of myelination, leukoencephalopathy and cerebral atrophy are observed across all groups and are nonspecific. Subcortical WM involvement is the main leukoencephalopathy pattern on the "definite" group. Cerebellar atrophy is more frequent than cerebral atrophy. The presence of lactate peak on MRS, in the absence of hypoxia, ischemia or infection, is an independent metabolic marker and can be used to enhance the diagnostic certainty in RC defects. A low NAA/Cr was observed in all groups and it is a nonspecific marker.

I-6
Biochemical and molecular diagnostics: how do we decide what gene(s) to investigate?

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Clinical phenotype, family history, muscle histochemistry and respiratory chain enzymology all provide useful clues for guiding studies to identify the causative gene in patients with mitochondrial disorders. However, in most cases these indicators have low predictive value. For example, complex I deficiency can be caused by mutations in at least eight nuclear-encoded subunit genes, six mtDNA-encoded subunit genes, and a substantial number of unidentified genes. Clearly, we need other methods to help guide molecular investigations, and two such approaches will be discussed. Blue Native PAGE immunoblotting is being used in an increasing number of labs as a supplementary method to enzyme diagnosis, and it appears that mutations in specific genes usually give consistent BN-PAGE results. In complex I deficiency, for example, mutations in some subunits such as ND6 and ND1 give gross diminution in the amount of fully assembled complex, while mutations in ND3 have relatively little effect on the BN-PAGE profile. Mutations in some nuclear-encoded subunits such as NDUFS4 result in loss of fully assembled complex I with accumulation of a large assembly intermediate of ~ 700 kDa. DNA microarrays are also an attractive way to attempt to predict the causative gene. Results with Affymetrix cDNA chips or custom-made cDNA "mitochips" have been promising but are expensive and prone to difficulty with interindividual variation. An alternative approach is to use cheaper Compugen oligo-arrays representing 19,000 genes, which recently allowed us to identify gross underexpression in two patient cell lines of a subunit gene not previously shown to cause complex I deficiency.

I-7
Molecular diagnostics: new developments

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The mitochondrial respiratory chain (RC) results from the expression of both mitochondrial and nuclear genes. The number of disease-causing mutations in nuclear genes is steadily growing and mtDNA deletions and mutations account for no more than 15–20% of patients. Unfortunately, for only very few patients have the disease causing mutations been identified. Thus, elucidating the genetic bases of RC is both essential for genetic diagnosis of patients and for fundamental knowledge of these disorders. The molecular diagnostics of mitochondrial disorders come under both genetic diagnostic and research. Indeed, identification of a new gene in a specific patient allows to perform genetic diagnosis in other families and identification of mutations in already known disease-causing genes allows to constitute a cohort of patients for further functional studies. Thus, elucidating the genetic bases of RC deficiency is an essential task that needs the use of several appropriate strategies. Fine phenotypage of patients and candidate gene screening is a first step for the constitution of well-characterized cohorts of patients. Genetic mapping has to be used in large families. This approach is greatly enhanced in the case of consanguineous families. The consanguinity of the parents should also lead to test genetic markers surrounding the gene loci rather than to directly sequence several candidate genes. However, the main problem is encountered in the cases of sporadic cases for which no genetic approaches can be developed. In these cases, functional complementation by human chromosomes or cDNA is the only presently available strategy.

O-4
Consensual protocols for the spectrophotometric assays of respiratory chain activities

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Mitochondrial disorders have very diverse clinical presentations. Genetic investigations are most often conducted after a first diagnosis based on metabolic alterations, morphological anomalies, and/or defects of the mitochondrial respiratory chain. For efficient screening, this body of arguments has to be searched for by comparable protocols. In France, the hospitals involved in the diagnosis of mitochondrial diseases use quite different protocols. We have evaluated the consequences of the diverse protocols by analysis of an identical sample. The results demonstrate the need to obtain consensual protocols in the prospect of multicentric recruitment of patients with similar defect. These protocols were chosen by discussion and then experimentally validated on beef heart mitochondria and on postnuclear supernatants from mouse and human muscle. The goal was to have reliable assays with (1) really maximum velocities (to better distinguish mild defects), (2) linear reaction with time (to avoid miscalculations), and (3) reaction rate proportional to the amount of tissue (to avoid false positive and false negative). This work demonstrates the difficulties of the respiratory chain assays. The complete set of experimental validations will be accessible on the website HC Forum. In general, the more active the activity, the more difficult to reach the proposed goal. Several unexpected behaviour were observed (particularly in complex I and complex III analyses). In order to set up a quality control, we have started to immortalize skin fibroblasts from patients expressing diverse defects of the respiratory chain. We have also started experimental validation of the consensual protocols on normal cultured skin fibroblasts.

O-5
Automated analysis of respiratory chain complex (RCC) enzyme activities in cultured skin fibroblasts

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We have developed an automated spectrophotometric assay in cultured skin fibroblasts to determine the following RCC activities: rotenone-sensitive NADH CoQ reductase (Complex I), rotenone-sensitive NADH-cytochrome *c* reductase (I + III), succinate cytochrome *c* reductase (II + III), and cytochrome *c* oxidase (IV). Citrate synthase (CS) is also measured as indicator of cellular integrity and mitochondrial proliferation, and for result normalization purposes. Skin fibroblasts are cultured in uridine-supplemented media, harvested, and diluted to 1 mg/ml protein. All five RCC components are analyzed simultaneously on a Hitachi 912 automatic analyzer. Enzyme activities are reported as nmol substrate/min/mg protein and mU/mg protein. Reference ranges were derived from the analysis of normal cell lines purchased from commercial sources. Intra-assay precision ($N = 10$) for each of the five RC profile assays yielded coefficients of variance ranging from 0% to 9%, with a mean of 3%. Seven disease controls including complex I, II + III, and IV deficiencies were blindly tested for validation. Concordant results were obtained in all but one case. One discrepant result was obtained from a cell line diagnosed elsewhere to have complex I deficiency. Our analysis showed normal activity (66% of mean control value) with mild CS elevation (141% of mean control value). If confirmed by additional studies, this discrepancy could be explained by a spontaneous recovery of activity over period time in cultured condition, a previously described event in RCC disorders. Advantages of this automated method are fast analytical time (less than 1 h), reduced sample size, and improved analytical precision.

I-8
New genes in mitochondrial disorders

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The identification of nuclear disease genes is a ''hot issue'' in mitochondrial medicine. We still ignore the genetic cause in more than 80% of infantile, and in approximately 50% of adult-onset, mitochondrial disorders. High-throughput, automated analysis of mtDNA has become available in several specialized centers worldwide, and helps exclude a primary involvement of the mitochondrial genome in these cases. The discovery of new disease genes is hampered by the remarkable clinical and genetic heterogeneity of mitochondrial syndromes and the scarcity of informative families. On the other hand, the development of new bioinformatics algorithms and the completion of the human genome project are new tools that, together with traditional strategies, can help progress in this area. An example is the recent discovery of the gene responsible for Ethylmalonic Encephalopathy (EE), a devastating infantile metabolic disorder affecting brain, gastrointestinal tract and peripheral vessels. Using an integration of physical and functional genomic data sets, we identified ETHE1 as a new gene, responsible for EE. The corresponding protein

product is targeted to mitochondria and internalized into the matrix after energy-dependent cleavage of a short leader peptide. The severe consequences of its malfunctioning indicate an important role of the ETHE1 gene product in mitochondrial homeostasis and energy metabolism. Similar strategies led us to identify new disease loci for different biochemical defects of the respiratory chain. Finally, the characterization of the clinical spectrum associated with mutations in individual genes is important for the establishment of diagnostic guidelines in mitochondrial disorders.

I-9

How to find genes for recessive oxidative phosphorylation disorders, and what to do once you have found them

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Most autosomal recessive oxidative phosphorylation disorders show up in the pediatric population, and they are nearly always fatal in the first years of life. In addition, there is a great deal of genetic heterogeneity associated with similar biochemical deficiencies, so gene discovery by conventional linkage analysis is usually not feasible. Primary skin fibroblast or myoblast cultures from these patients often exhibit a biochemical phenotype, and it is therefore possible to contemplate identifying the underlying genetic defects by functional complementation techniques such as transduction with retroviral expression libraries, microcell-mediated chromosome transfer, or overexpression of candidate genes using retroviral vectors. I will discuss our efforts along these lines in patients with isolated Complex I and Complex IV defects and in patients with multiple respiratory chain enzyme deficiencies. I will also show how studies in cell lines and tissues from patients who have mutations in assembly factors or structural subunits have contributed to our understanding of the assembly of the respiratory chain complexes.

O-6

Truncated product of the bifunctional DLST gene involved in the assembly of the mitochondrial respiratory complexes and associated with Alzheimer's disease

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Dihydrolipoamide succinyltransferase (DLST) is a subunit-enzyme of the alpha ketoglutarate dehydrogenase complex involved in the Krebs cycle. We previously reported a genetic association between polymorphisms of the DLST gene and Alzheimer's disease (AD) [Lancet 350 (1977) 1367]. While studying how the DLST genotype contributes to the pathogenesis of AD, we detected a novel mRNA that is transcribed starting from intron 7 and its translational product (designated MIRTD). MIRTD localized to the intermembrane space of mitochondria. To investigate the function of MIRTD, we established human neuroblastoma cell lines expressing a kind of ribozyme that specifically digests the MIRTD mRNA. The expression of the ribozyme specifically eliminated the MIRTD protein and the resultant MIRTD-deficient cells exhibited a marked decrease in the amounts of subunits of the complexes I and IV, resulting in a decline of the respiratory activity. A pulse-label experiment revealed that the loss of the subunits is a posttranslational event. Thus, the DLST gene bifunctionally contributes to mitochondrial energy metabolism: the full-length DLST protein is involved in the Krebs cycle in the matrix while the truncated version, MIRTD, contributes to the assembly of the respiratory complexes in the intermembrane space. In addition, MIRTD expression was decreased depending upon the AD-associated polymorphism of the DLST gene. Moreover, the MIRTD-mRNA was not detected in the brains isolated from half of AD patients. Several laboratories have reported the association between AD and defects of complex IV. Therefore, decrease of MIRTD may contribute to AD by affecting the biogenesis of the mitochondrial respiratory chain.

O-7

Pyruvate dehydrogenase E1 beta subunit deficiency

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Pyruvate dehydrogenase deficiency is clinically highly heterogeneous, with presentations including neonatal lactic acidosis, Leigh syndrome and chronic neurodegeneration. The pyruvate dehydrogenase complex is composed of multiple copies of five main subunits, the E1 alpha and E1 beta subunits of the E1 (pyruvate dehydrogenase) component, E2 (dihydrolipoamidetransacetylase), E3 (dihydrolipoamide dehydrogenase) and the E3 binding protein. The clinical heterogeneity does not simply reflect underlying genetic variability as the great majority of cases are due to mutations in the X-linked gene for the E1 alpha subunit. A small number of patients have mutations in the E3 and E3 binding protein genes, but no mutations have previously been defined in the genes for either the E1 beta or E2 subunits. We describe two unrelated patients with pyruvate dehydrogenase deficiency due to mutations in the gene for the E1 beta subunit. Both patients presented with lactic

acidosis and muscle hypotonia in infancy and had marked reduction in enzyme activity and immunoreactive E1 beta protein. In each case there was a missense mutation (Y132C and P344S), which is predicted to reduce the stability of the tetrameric, alpha2-beta2, E1 enzyme. Activity of the pyruvate dehydrogenase complex was restored in cultured fibroblasts from both patients by transfection and expression of the normal E1 beta coding sequence.

I-10
Structural proteomics of mitochondria

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Almost all of the major components of mitochondrial inner membranes were identified before the proteomic age. Reanalysis of these membranes by mass spectrometric methods has confirmed the presence of its protein components, but the identification of some important proteins required careful detailed analysis. Other components that were known to be present, including hydrophobic proteins, small proteins and highly charged proteins, for example, were missed. It is likely that many less abundant mitochondrial membrane proteins were also missed and that they remain to be discovered. Many of the proteins that were detected contain posttranslational modifications that have been defined by detailed analysis, and some of these modifications are transient. Other proteins are known to be present, but their biological functions are unknown. Thus, the definition of the mitochondrial membrane proteome is far from complete.

The determination of the structural proteome of the respiratory chain is well advanced. High-resolution structures have been solved for complexes II, III and IV, and the structural analysis of ATP synthase is at an advanced stage. The structural analysis of complex I and of the transport proteins that support respiration is just beginning.

The lecture will review the achievements and the problems that remain to be solved in establishing the structural proteome of mitochondrial membranes.

I-11
Replication of mitochondrial DNA

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Mitochondrial DNA (mtDNA) of humans and other mammals was long believed to replicate via a strand-asynchronous mechanism, which entailed continuous synthesis of both strands of DNA from physically and temporally distinct sites. Replication by such a mechanism would inevitably give rise to molecules with extensive single-stranded regions. We have demonstrated that the majority of replicating molecules are double-stranded in carefully prepared mtDNA samples, and that single-stranded regions arise as artefacts due to RNase degradation. This is because mtDNA replication frequently involves wholesale ribonucleotide incorporation on one strand.

In parallel studies, we have found that replication initiates not from a discrete unidirectional origin, as was previously supposed, but bidirectionally from multiple sites dispersed across a broad zone. Unlike the *E. coli* chromosome, the mtDNA terminus is not opposite the origin (initiation zone), hence one replication fork arrests much earlier than the other, thereafter giving the illusion of unidirectional replication. Replication of mtDNA generally terminates in the major non-coding region.

I will describe new data which suggest the centre of the initiation zone maps to the ND 6 gene and that initiation can occur either side of the displacement loop.

I-12
Mammalian mitochondrial transcription and DNA replication: in vitro reconstitution and molecular characterization

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Mammalian mtDNA only contains two major promoters, the light- and heavy-strand promoters (LSP and HSP), which produce near-genomic length transcripts that after RNA processing release individual mRNAs, tRNAs and rRNAs. We have previously identified two transcription factors, transcription factors B1 (TFB1M) and B2 (TFB2M), and demonstrated that they can each support promoter-specific mtDNA transcription in a pure recombinant in vitro system containing mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM). Our current studies demonstrate that RNA polymerse specifically recognizes sequence elements localized between nt -1 and -14 relative to the transcription start site, much similar to what has previously been described for the homologous RNA polymerase encoded by bacteriophage T7.

Transcription from LSP is not only necessary for gene expression but also for production of RNA primers required for initiation of mtDNA replication origin of leading-strand replication (OH). We will here describe our current studies of replication primer formation and attempt

to reconstitute OH-dependent initiation of mtDNA replication in vitro.

O-8

The majority of TFAM molecules are required for maintenance of mitochondrial DNA and a minority for transcription: TFAM-titration model

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Mitochondrial transcription factor A (TFAM), a transcription factor for mitochondrial DNA (mtDNA), is a member of the high mobility group proteins and also possesses properties of nonspecific DNA-binding. Because replication of mtDNA is proposed to be coupled with transcription, TFAM is thought to be essential for replication of mtDNA. To clarify a role of TFAM in the maintenance of mtDNA, we varied the amount of TFAM in HeLa cells using a Tet-off gene expression system and RNA interference. The amount of mtDNA changed in direct proportion to that of TFAM, showing the amount of mtDNA is precisely determined by the amount of TFAM. TFAM lacking the C-terminal 25 amino acids (C-tail), which does not activate transcription, was as effective as full-length TFAM, suggesting that the transcription activity of TFAM does not mainly contribute to the determination of the amount of mtDNA. When we knocked down endogenous TFAM to about 15% by RNA interference while maintaining the amount of mtDNA by expressing the C-tail-deleted TFAM, the mitochondrial transcription rate was maintained at a control level, suggesting that TFAM is in excess for transcription but is dose-limiting for the determination of the amount of mtDNA. Based on the findings that (1) the recombinant TFAM as well as endogenous TFAM are mostly bound to mtDNA, (2) TFAM is abundant enough to cover the entire region of mtDNA, and (3) TFAM is dose-limiting for the amount of mtDNA, we propose a model that TFAM titrates the mass of mtDNA directly.

O-9

Organization and dynamics of human mitochondrial DNA

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Heteroplasmic mutations of mitochondrial DNA (mtDNA) are an important source of human diseases. The mechanisms governing transmission, segregation and complementation of heteroplasmic mtDNA-mutations are unknown but depend on the nature and dynamics of the mitochondrial compartment as well as on the intramitochondrial organization and mobility of mtDNA. We show that mtDNA of human primary and immortal cells is organized in several hundreds of nucleoids that contain a mean of two to eight mtDNA molecules each. Nucleoids are enriched in mitochondrial transcription factor A and distribute throughout the entire mitochondrial compartment. Using cell fusion experiments, we demonstrate that nucleoids and respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA. In contrast, nucleoid mobility was lower within mitochondria of mtDNA-containing cells, as differently labeled mtDNA molecules remained spatially segregated in a significant fraction (37%) of the polykaryons. These results show that fusion-mediated exchange and intramitochondrial mobility of endogenous mitochondrial components are not rate-limiting for intermitochondrial complementation but can contribute to the segregation of mtDNA molecules, and thus of mtDNA mutations, during cell growth and division.

I-13

The chaperone/Tom70 mitochondrial targeting pathway

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Most mitochondrial proteins are synthesized on ribosomes in the cytosol, and imported into the organelle after translation. In mammals, many proteins of the inner membrane metabolite carrier protein family are bound by the cytosolic chaperones Hsc70 and Hsp90 during their targeting. Docking of the chaperones onto the Tom70 import receptor is important for the transfer of the carrier preproteins to the translocation machinery of the mitochondrial outer membrane. Disruption of the interaction between the chaperones and Tom70 interferes with the import of Tom70-dependent preproteins. Also, inhibition of either Hsc70 or Hsp90 activity diminishes the import efficiency of these preproteins. In the cytosol, these preproteins are maintained in high-molecular-weight chaperone complexes similar to those active in the folding of cytosolic polypeptides. All of the components required for mitochondrial targeting are contained in the chaperone complexes. Thus, the Hsc70 and Hsp90 chaperone system responsible for polypeptide folding also

participates in the distinct but related process of protein targeting.

I-14
Regulation of mitochondrial activity by proteolysis

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The biogenesis and homeostasis of mitochondria is controlled by conserved and ubiquitous ATP-dependent proteases residing within the organelle. The identification of a disease-causing mutation in paraplegin has recently linked a dysfunction in the mitochondrial quality control system to axonal degeneration in hereditary spastic paraplegia (HSP). Paraplegin represents a subunit of a highly conserved ATP-dependent AAA protease in the inner membrane of mitochondria. Two AAA proteases built up of homologous subunits and exposing catalytic sites to opposite membrane surfaces are apparently ubiquitously present in mitochondria of eukaryotic cells. The m-AAA protease is active on the matrix side and is composed of Yta10 and Yta12 subunits in *S. cerevisiae* and of Afg3L2 and paraplegin subunits in human mitochondria. Complementation studies in yeast revealed functional conservation of mitochondrial AAA proteases. m-AAA proteases are crucial for mitochondrial activity in yeast and human cells. Yeast cells lacking the m-AAA protease exhibit deficiencies in the expression of mitochondrially encoded polypeptides and the posttranslational assembly of respiratory chain complexes. Similarly, HSP fibroblasts show deficiencies in complex I activity and resistance towards reactive oxygen species. Ongoing studies to characterise the cellular function of the conserved proteolytic system within mitochondria will be discussed.

O-10
Chaperone-like activities of a new cytosolic factor AIP and import receptors Tom20 and Tom22 facilitate mitochondrial protein import

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Most mitochondrial proteins are synthesized in the cytosol with N-terminal presequences, maintained in a loosely folded import-competent conformation by cytosolic chaperones, and are imported into mitochondria via translocator complexes containing preprotein receptors Tom20 and Tom22. We identified arylhydrocarbon receptor interacting protein (AIP), a cytosolic factor which interacts with both Tom20 and preproteins [J. Cell Biol. 163 (2003) 45]. In vitro import assay showed that AIP prevents pre-ornithine transcarbamylase (pOTC) from loss of import-competency. In cultured cells, overexpression of AIP enhanced pOTC import, and its depletion by RNA interference impaired the import. In vitro binding assay revealed that AIP as well as Tom20 preferentially bind to mitochondrial preproteins. Formation of a ternary complex of Tom20, AIP and preprotein was also observed. These results indicate that AIP functions as a cytosolic factor that mediates preprotein import into mitochondria. Furthermore, aggregation suppression assay using citrate synthase showed that both AIP and Tom20 have chaperone-like activities to prevent this protein from aggregating [J. Biol. Chem., in press]. Tom20 binds to guanidinium chloride-unfolded substrate proteins regardless of the absence of presequence. Overexpression of Tom20 suppressed the cytosolic degradation of pOTC. Tom22 also showed a similar aggregation suppression activity, whereas Tom70 did not. Together, these results suggest that AIP, Tom20 and Tom22 have chaperone-like function to prevent their substrate preproteins from degradation in the cytosol and on the mitochondrial surface, and facilitate their import into the organelle.

O-11
Biogenesis of the mitochondrial inner membrane

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The mitochondrion has two pathways for the import of proteins coded in the nucleus. The general import pathway is utilized by precursors with an amino-terminal targeting presequence. Precursors are first translocated across the Translocase of the Outer Membrane (TOM) and then through the Translocase of the Inner Membrane(TIM23). From our work, we identified a second import pathway for the import and insertion of inner membrane proteins such as those in the mitochondrial carrier family and the import components. This import pathway is referred to the TIM22 pathway. Components include a family of small Tim proteins in the mitochondrial intermembrane space, Tim8p, Tim9p, Tim10p, Tim12p, and Tim13p, and the inner membrane proteins, Tim22p, Tim18p, and Tim54p. Protein import is an essential function for yeast and most of these genes are required for viability. Mutations in the human homolog of Tim8p, DDP1 (Deafness/Dystonia protein), cause the X-link Mohr–Tranebjaerg syndrome (MTS). Symptoms include blindness, deafness, dystonia, and mental deterioration. We have shown that the Tim8p–Tim13p complex fails to assemble in fibroblasts derived from

patients. From biochemical studies in yeast, we have shown that DDP1 mediates the import of Tim23p. A decreased abundance of these translocators may be the underlying cause of MTS.

I-15
Mouse models of isolated cytochrome oxidase deficiency

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We have developed mouse models of cytochrome *c* oxidase deficiency by the conditional deletion of the COX 10 gene, which is necessary for the formation of heme A in mitochondria.

Deletion of COX 10 in the CNS by the synapsin-Cre led to death on days P9–P12, in a model resembling Leigh syndrome. Immunohistochemical studies showed the presence of cortical neurons with reduced COX I and increased SDH(Fp). COX enzyme activity in KO brain homogenates was decreased by 20%.

Deletion of COX 10 in the CNS by the CamKIIa-Cre led to a neurological phenotype starting at age P105–P120. These mice exhibited altered behavior with excited/maniac phases interspersed with inactive/unresponsive phases. The phenotype worsened in the course of 4–5 weeks leading to death. Brain homogenates from mice at P120 showed a drastic reduction in COX I, but normal levels of subunits belonging to other OXPHOS complexes, suggesting that neurons with no or low COX were still present in knockout mice brains. COX enzyme activity in KO brain homogenates was decreased by 70–90%.

Deletion of COX 10 by the myosin light chain-Cre led to a myopathy with patchy distribution of COX negative and positive segments. This pattern is due to the incomplete deletion of both COX 10 alleles in some nuclear domains. The myopathy had onset at P90, leading to death at P120–180. COX activity in P30 mouse muscle mitochondria was 20% of controls. Muscle mitochondria of P120 animals had less than 5% of the normal COX activity.

I-16
Molecular genetics of complex I-deficient Chinese hamster cell lines

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Our laboratory has described a series of Chinese hamster cell mutants with very severe complex I deficiencies. Defects in three X-linked genes had been proposed, and two of these genes have now been identified. The third may be defective in an assembly factor or an as yet unidentified subunit. One group of mutants is defective in the NDUFA1 gene encoding the MWFE subunit. Various mutant alleles have been characterized, including a null mutant. We have recently described an inducible system in which MWFE synthesis is under the control of an inducible promoter. Such a system promises to be useful to study the assembly of complex I. Studies on the import and orientation of this integral membrane protein will be described: it has an atypical mitochondrial targeting sequence that is not processed after import. A second group of mutants has been characterized with chain-terminating codons in the gene for the ESSS subunit. This subunit is also absolutely required for complex I assembly and activity.

Our null mutants can be complemented with cDNA constructs encoding wild-type or modified MWFE or ESSS subunits epitope-tagged with either HIS or HA. Such epitope-tagged proteins can be expressed in a wild-type background and compete with the endogenous subunit for assembly. The epitope-tagged subunits are useful in cross-linking studies to determine protein–protein interactions in the integral membrane subcomplex, and even in the various mutants in which complex I assembly is incomplete.

O-12
Alternative topogenesis of Mgm1 is essential for the integrity of mitochondrial structure

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Mitochondrial morphology and inheritance of mitochondrial DNA (mtDNA) in yeast depend on the dynamin-like GTPase Mgm1. Mgm1 is present in two isoforms in the intermembrane space of mitochondria. The large isoform (l-Mgm1) is anchored to the inner membrane with an N-terminal hydrophobic segment which is absent in the short isoform (s-Mgm1). s-Mgm1 is generated by proteolytic cleavage of l-Mgm1 by the inner membrane rhomboid-type protease Pcp1. Both the deletion of MGM1 and of PCP1 result in fragmentation of mitochondria and loss of mtDNA. We show that the deletion phenotype of PCP1 is a direct consequence of the absence of s-Mgm1 and that both isoforms of Mgm1 are required for its function. Near its N-terminus Mgm1 contains two conserved hydrophobic segments. The more C-terminal one is cleaved by Pcp1. However, changing the hydrophobicity of the N-terminal one influences the ratio of

both Mgm1 isoforms. Furthermore, the generation of s-Mgm1 and mitochondrial morphology depend on the ATP level in the matrix. Based on these data, we present a model of alternative topogenesis, which ensures the balanced formation of both Mgm1 isoforms. This model suggests how on a molecular level the ATP concentration in the matrix may influence mitochondrial morphology.

O-13
COX assembly: Mss51p and Cox14p jointly regulate Cox1p expression in the yeast *S. cerevisiae*

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Mutations in SURF1, the human homolog of yeast SHY1 are responsible for Leigh syndrome, a neuropathy associated with cytochrome oxidase (COX) deficiency. Previous studies of the yeast model of this diseaseshowed that mutant forms of Mss51p, a translational activator of Cox1 mRNA, partially rescue the COX deficiency of shy1 mutants by restoring normal synthesis of the mitochondrially encoded Cox1p subunit of COX.

Here we present evidence showing that the synthesis of Cox1p, measured in vivo, is reduced in most COX mutants but is restored to that of wild type by the same mutation that suppresses shy1 mutants. An important exception is a null mutation in COX14, which by itself or in combination with other COX mutations does not affect Cox1p synthesis. Cox14p and Mss51p are shown to interact with Cox1p and with each other. We propose that the Cox14p-dependent interaction of Mss51p with Cox1p, to form a transient Cox14p–Cox1p–Mss51p complex, functions to down-regulate Cox1p synthesis. The release of Mss51p from the complex occurs at a downstream step of the assembly pathway, probably catalyzed by Shy1p.

I-17
Respiratory chain defects: what do we know for sure about their consequences in vivo?

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Respiratory chain deficiency can be the source of a multitude of cellular malfunctions which relative incidence on observed cellular phenotypes and clinical features remains to be established in most cases. This basically reflects the chief importance of mitochondrial activities in any living cell and the high degree of integration of both metabolic and signalling pathways where mitochondria play often a key role. In practice, the ignorance of the actual consequences of respiratory chain defects certainly often hampers our ability to possibly counteract their presumably deleterious, but yet poorly defined effects. Indeed, at least from a theoretical point of view, the consequences of any respiratory chain defect constitute a quasi-endless list, which can be somewhat subcategorized according to their presumed mechanism. However, much less has been experimentally established in vivo. The scarcity of available material at least in human, the various severity and nature of the defects, the tissue to tissue differences, and the high number of interacting factors make it difficult to reach unequivocal conclusions. The aim of the presentation will be to review the few established consequences of respiratory chain defects, in situ and in vivo, and to examine their potential link with recognized clinical features associated with respiratory chain deficiencies.

I-18
Dying mitochondria in agonizing cells

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In biochemical terms, apoptosis relies on mitochondrial membrane permeabilization (MMP) and/or caspase activation of caspases. Apoptosis-inducing factor (AIF) is a flavoprotein with NADH oxidase activity normally contained in the mitochondrial intermembrane. Upon apoptosis induction, AIF translocates from mitochondria to the cytosol and to the nucleus. We have performed an extensive search for AIF-binding proteins, by screening for AIF partners that may enhance or inhibit its apoptogenic function. Cyclophilin A (CypA) was determined to interact with apoptosis inducing factor (AIF) by mass spectroscopy, co-immunoprecipitation, pull-down assays, and molecular modeling. Recombinant AIF and CypA proteins synergized in vitro in the degradation of plasmid DNA, as well as in the capacity to induce DNA loss in purified nuclei. AIF mutants lacking the CypA-binding domain were inefficient apoptosis sensitizers. Moreover, AIF failed to sensitize CypA knockout cells to apoptosis induction, and this defect in the AIF response was reversed by reintroduction of the CypA gene into CypA-deficient cells. Thus, AIF and CypA collaborate in apoptotic chromatinolysis. The principal AIF-inhibitory protein turned out to be Hsp70, an established apoptosis inhibitor. The Hsp70-binding domain of AIF does no overlap with its CypA binding domain. Hsp70 inhibits

AIF by preventing its mitochondrio-nuclear translocation. Inhibition of the interaction between Hsp70 and AIF by an AIF-derived decoy of Hsp70 (AAD70) facilitates the nuclear translocation of endogenous AIF in Hsp70 expressing cells and sensitizes a number of cancer cell lines to cell death induction.

O-14
Mutation-specific mitochondrial remodeling in complex I-deficient patient fibroblasts

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In this study we investigated cellular consequences of mutations in nuclear-encoded subunits of complex I (CI) of the respiratory chain. For this purpose we correlated mitochondrial shape and number, the expression profile of key mitochondrial proteins in human fibroblast cell lines derived from 4 healthy subjects and 13 patients with an isolated CI deficiency. Computer-assisted analysis of mitochondrial morphology revealed distinct mutation-dependent aberrations in mitochondrial morphology. These aberrations were independent of cell cycle phase and were abolished during complementation assays. Two distinct classes of mutations were distinguished, one in which mitochondrial number and degree of branching were proportional to CI activity (class I) and another where these two parameters were negatively or uncorrelated with CI activity (class II). For both classes, total expression of the CI-39 kDa subunit correlated linearly with CI activity and the amount of CI-39 in fully assembled CI. These findings indicate the presence of a general adaptive mechanism, which combines mitochondrial biogenesis with increased expression of CI and, consequently, CI activity. We conclude that the effectiveness of this mechanism in counterbalancing CI deficiency depends on the nature of the mutation.

O-15
Miro, a novel GTPase with Ca^{2+}-binding EF-hand motifs, may integrate cell-signaling pathways with mitochondrial dynamics

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Although mitochondria are known to be dynamic organelles, changing size, shape and distribution in response to cellular signals, a protein that senses such signals and modulates mitochondrial dynamics has not been identified. A genomic search for human Rho-type GTPases revealed Miro (for mitochondrial Rho), an evolutionarily conserved protein family with a unique domain structure: two tandem Ca^{2+}-binding EF-hand motifs flanked by two GTPase domains. Studies in mammalian cells raised the possibility that Miro may be involved in mitochondrial homeostasis and apoptosis.

To understand the role of Miro in mitochondrial dynamics and Ca^{2+}-sensing, we initiated studies on Ymr1p (yeast Miro) in *S. cerevisiae*. We found that deletion of the YMR1 gene altered wild-type mitochondrial tubular networks. Mitochondria in cells lacking Ymr1p form collapsed tubules, grape-like clusters and distorted spheres. These morphological changes are not due to defects in mitochondrial fission or fusion, as neither process is abolished in the ymr1 null mutant. In addition, inner mitochondrial membrane cristae formation occurs in abnormally shaped organelles. Microscopic and biochemical fractionation studies localized Ymr1p in the outer mitochondrial membrane as a tail-anchored protein with its N terminus exposed to the cytoplasm. Moreover, mutational analysis indicated that both of the GTPase domains and the EF-hand motifs are important for Ymr1p function. These observations suggest that Ymr1p is a mitochondrial transmembrane GTPase required for mitochondrial morphology maintenance. Studies are underway to determine whether and how Miro integrates intracellular Ca^{2+}-signaling events with mitochondrial dynamics.

I-19
Mitochondrial disease in flies

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The *Drosophila* mutant tko, affecting the mitochondrial protein synthetic apparatus, exhibits respiratory chain deficiency and a phenotype resembling various features of mitochondrial disease in humans (paralytic seizures, deafness, developmental retardation). We are using this mutant to analyse the cellular and genomic targets of mitochondrial dysfunction, and to identify ways in which the phenotype can be alleviated.

Transgenic expression of wild-type tko in different patterns in the mutant background reveals critical times and cell-types for production of components of the mito-

chondrial disease-like phenotype. Mitochondrial bioenergy deficit during the period of maximal growth, as well as in different parts of the nervous system, appears to be most deleterious.

Inbreeding of tko mutant lines results in a systematic improvement in all phenotypic parameters tested. We report the first results of genetic mapping and transcriptomic analysis, which gives clues as to the genes and pathways that can modify mitochondrial disease-like phenotypes in a model metazoan.

O-16
A switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts

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We investigated temporal changes occurring during development and progression of respiratory chain dysfunction in mice with tissue-specific knockout of mitochondrial transcription factor A (Tfam) in the heart.

Mitochondrial DNA levels were reduced to $\sim 25\%$ in knockout mouse hearts at 2 weeks of age. The heart size of knockout mice increased progressively starting at 3 weeks of age until death occurred at $\sim 10-12$ weeks of age. Knockout mice developed a severe decline in respiratory chain enzyme activities in the heart and a simultaneous reduction of mitochondrial ATP production rate starting ~ 2 weeks of age. Mitochondrial mass was normal until a late stage of disease when an increase of $\sim 25\%$ was observed.

Studying gene expression with microarrays, we found a coordinate down-regulation of genes encoding fatty acid oxidation enzymes in knockout hearts, whereas several genes encoding glycolytic enzymes were up-regulated. During the fetal stages the mammalian heart mainly relies on glucose as an energy substrate. Immediately after birth the expression of genes that are involved in fatty acid oxidation is induced and the adult heart predominantly uses fatty acids as a source for energy production. Our findings are consistent with reactivation of the fetal gene expression pattern, a common phenomenon during different forms of cardiomyopathy. In the Tfam knockout mice this switch occurred at an early stage of disease before the increase of mitochondrial mass. It is unlikely that this response is beneficial for optimal function of the heart and may instead be a contributing factor to the progression of mitochondrial cardiomyopathy.

O-17
Modelling mitochondrial complex I disorders in the nematode *Caenorhabditis elegans*

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Mitochondrial dysfunction, with an estimated incidence of 1 in 10,000 live births, is among the most common genetically determined conditions. Missense mutations in the human NDUFV1 gene, which encodes the 51-kDa active site subunit of the NADH-ubiquinone oxidoreductase or complex I, can lead to severe neurological disorders. Owing to the rare and often sporadic nature of mitochondrial disorders, the mechanisms of pathogenesis of most mutations remain poorly understood. We have generated transgenic strains of *Caenorhabditis elegans* that express disease-causing mutations in the nuo-1 gene, the *C. elegans* homologue of NDUFV1. The transgenic strains demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent mitochondrial respiration. Furthermore, mutant strains are hypersensitive to exogenous oxidative stress. The lactic acidosis induced by the NDUFV1 mutations could be partially corrected with the vitamins riboflavin and thiamine, or with sodium dichloroacetate, an activator of the pyruvate dehydrogenase complex, resulting in significant increases in animal fitness. Surprisingly, cytochrome *c* oxidase activity and protein levels were reduced, establishing a connection between complexes I and IV. Our results indicate that complex I mutations exert their pathogenic effects in multiple ways: by impeding the metabolism of NADH, by increasing the production of reactive oxygen species, and by interfering with the function or assembly of other mitochondrial respiratory chain components.

O-18
Gene expression profiles define mitochondrial pathology in yeast mutants and human disease

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Gene expression profiling and data analysis were performed in yeast and human mutants of the mitochondrial system. Mathematical techniques were derived from

basic statistics, clustering, discriminant analysis and principal component analysis (PCA). Background knowledge was incorporated for the yeast data by downloading website ontology annotations automatically. We used the set from Hughes et al. [Cell 102 (2000) 109; or http://www.rii.com] containing whole genome expression profiles of 300 gene knockout experiments in yeast. PCA reduced the dimension of the data set significantly without losing much of the information and reduced noise as well. By subsequent discriminant analysis, we could distinguish mitochondrial gene knockouts from non-mitochondrial gene knockouts (cytoskeleton, cell membrane, cell wall, cytoplasm, lysosome/vacuole/peroxisome). As a next step, we applied the same approach on Affymetrix GeneCHIP data (U133A) of a group of 18 fibroblast cell lines of patients with Leigh disease, caused by a Surf1-mutation and 11 controls. The Surf1-patients could be reliably distinguished from the controls by the expression changes of only three genes. It is not clear at this point if these classifiers are generally applicable and reflect Surf1-specific pathology or are a common characteristic of mitochondrial pathology. Gene expression studies in additional Surf1-patients and patients with mtDNA caused Leigh and MELAS syndrome and with secondary mitochondrial pathology are currently being performed to solve this question. As differences in gene expression in fibroblasts were not very explicit, we are also testing muscle biopsy specimens to identify pathogenic genetic pathways in mitochondrial disease.

I-20
Immunological and proteomics approaches to characterisation of mitochondrial diseases

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Diagnosis of genetic mitochondrial diseases remains difficult. Similarly, analysis of the role of OXPHOS in late onset conditions such as Parkinsons disease, Alzheimers disease and NIDDM is limited by a lack of straightforward approaches for isolating the respiratory chain complexes and ATP synthase for detailed study of amounts, specific activities and posttranslational modifications.

We have now obtained monoclonal antibodies, which can isolate each of the five OXPHOS complexes in active form and fully assembled. We show how these mAbs can be used to quantitate the levels of the different complexes in as little as 10 μg of mitochondria and how posttranslational modifications including oxidative damage and phosphorylation can be determined by mass spectrometry on equally low amounts of patient material.

O-19
Integrative analysis of the mitochondrial proteome

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A broad range of genome-wide studies involving mitochondrial proteins have been accumulated recently. These include expression profiling, deletion phenotype screening, large-scale localisation studies, protein–protein interactions and proteomic studies by mass spectrometry to name but a few. While their information content is often complementary, sensitivity and specificity of different methods to detect mitochondrial proteins vary greatly. The integration of these systematic studies allows the prediction of candidates with high accuracy and provides a way of benchmarking individual high throughput (HT) experiments.

In a first step, we integrated 22 data sets from HT approaches with relevance for mitochondria in the online database MitoP2. For the evaluation of sensitivity and specificity of the various experiments, we generated a reference set of mitochondrial proteins based on direct evidence in single gene studies. The comparison of different approaches to characterize the mitochondrial proteome reveals strengths and weaknesses of the approaches. Using the collected data, we developed a new strategy for the prediction of organellar proteins. A Support Vector Machine was trained with two data sets covering the reference set ($n = 477$) and a set of non-mitochondrial proteins ($n = 300$). The Leave-One-Out estimation of the SVM prediction revealed a precision of 90%. Altogether SVM predicts 200 new candidates. Each new candidate was identified in several HT approaches, indicating saturation of the mitochondrial parts list. Most proteins were identified by HT localisation experiments, followed by proteomics and deletion phenotype screens. By providing new mitochondrial candidate proteins, the MitoP2 database will help to improve the genetic analysis of human mitochondriopathies.

I-21
Mitochondria and cancer

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The key role of mitochondria in cancer is well established; e.g. their involvement in apoptotic mechanisms is of great importance. Recently, a new link between mitochondria and cancer has been introduced. We have shown that heterozygous dominantly inherited inactivating mutations in fumarate hydratase (FH) gene predispose to uterine and cutaneous leiomyomatosis, leiomyosarcoma, and renal cell cancer (hereditary leiomyomatosis and renal cell cancer, HLRCC). Succinate dehydrogenase (SDH) defects have previously been shown to predispose to paragangliomas, and tricarboxylic acid cycle (TCAC) defects are a known cause for recessive neurodegenerative disorders. We have now found common ground for the FH and SDH phenotypes by identifying SDHB as a new renal cell cancer predisposition gene. These studies have revitalized the field of TCAC research in view of cancer.

However, it is unknown how FH and SDH defects cause tumor predisposition. Furthermore, the presence of an unknown genetic modifier appears to be necessary—at least in the case of FH defects—to promote progression from benign tumors to cancer. Possible tumorigenic mechanisms involved include hypoxia response, induction of oxidative stress, and DNA damage. Previously unknown functions not connected to energy metabolism may also exist and should be searched for.

Our further work is focused on characterization of the clinical tumor phenotype in FH mutation families, identification of the modifier gene required for the malignant phenotype, and functional studies on mutant and wild-type fumarase in tumor cell lines.

O-20
Succinate dehydrogenase and mitochondrial signalling in HIF1a stabilization

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Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor consisting of HIFa and HIFb subunits, which mediates adaptive responses to changes in tissue oxygenation. The oxygen-regulated subunit HIFa is stabilized in cells exposed to hypoxia. HIFa is negatively regulated at the level of protein turnover by proline hydroxylation, which mediates von Hippel–Lindau protein binding, ubiquinylation and proteasome degradation. Proline hydroxylation is catalyzed by prolyl-4-hydroxylases (PHD), oxygen and Fe^{2+}-requiring dioxygenases which decarboxylate 2-oxoglutaric acid to produce succinate. We hypothesized that inhibition of succinate dehydrogenase (SDH) using RNAi's targeted to SDH subunits B and D would lead to an accumulation of succinate in the cytosol which would then inhibit PHD and stabilize HIFa. Western blots using an anti-HIF1a antibody showed that HIF1a is stabilized by transient transfection with SDH RNAi in HEK293 and H1299 cells under normoxic conditions. SDH activity measured as TTFA-sensitive succinate-DCIP oxidoreductase is decreased in RNAi-transfected HEK293 cells. GC-MS analysis demonstrated that succinate concentrations are significantly elevated in cell extracts prepared from RNAi-treated cells. Moreover, HIF1a levels are increased in cells treated with dimethylsuccinate, which directly elevates succinate levels. These results demonstrate that inhibition of SDH elevates cellular succinate levels and stabilizes HIF1a, indicating that mitochondria can generate signals, which may enhance tumour progression by favoring adaptations to growth under hypoxic conditions.

I-22
Reconstructing proto-mitochondrial metabolism

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Mitochondria are ATP-producing eukaryotic organelles derived from the endosymbiosis of an alpha-proteobacterium. Using large-scale phylogenetic analyses to detect eukaryotic proteins that are phylogenetically closely related to alpha-proteobacterial proteins, we have reconstructed the proteome of the ancestral mitochondrion, the proto-mitochondrion. Based on the 630 proteins in this proteome, we subsequently derive its metabolism, which is geared towards energy conversion with the oxidation of fatty acids and glycerol as the main catabolic pathways. Comparing the reconstructed proto-mitochondrial proteome with current human and yeast mitochondrial proteomes reveals that the transition from endosymbiont to modern organelle was accompanied by major changes not only in the distribution of functional groups but also in their composition. The metabolism got more biased towards energy conversion and protein synthesis by diminishing functional classes such as amino acid and nucleotide metabolism, and losing others like carbohydrate metabolism and transport or cell envelope biogenesis. Some of these pathways have, however, not been lost from the cell, and have instead moved to other locations within it. This retargeting process has affected more than two thirds of the proto-mitochondrial derived proteins encoded in yeast and human genomes.

1. Gabaldon, T. and Huynen, M.A. (2003) Science, 301, 609.

I-23
***Yarrowia lipolytica*, a model organism to study mitochondrial complex I by yeast genetics**

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We have established the obligate aerobic yeast *Yarrowia lipolytica* as a powerful new model for the structural and functional analysis of mitochondrial complex I. All relevant yeast genetic tools have been developed in recent years for this nonconventional yeast and the genome has been sequenced. Complex I is an essential enzyme in *Y. lipolytica*. Nevertheless, complex I deletion strains could be generated by attaching the targeting sequence of complex I subunit to an external "alternative" NADH-dehydrogenase (NDH2) present in this yeast. Thereby, we could complement complex I deficiency by redirecting NDH2 to the matrix side. Deletion strains for several complex I subunits have been constructed that can be complemented by shuttle plasmids carrying the deleted gene. This allows for efficient site-directed mutagenesis of individual subunits of *Y. lipolytica* complex I and was used to reconstruct and characterize several pathogenic mutations in the PSST, TYKY and 24-kDa subunits of complex I that cause severe neuromuscular disorders in man. Yellow fluorescent protein was attached to the carboxy-terminus of the 30-kDa subunit and allows in vivo localization of complex I within the *Y. lipolytica* mitochondria.

I-24
Creation of mtDNA mutator mice

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It has been extensively documented that the normal ageing process in humans, monkeys and rodents is associated with accumulation of low levels of point mutations and deletions of mtDNA. The role of somatic mtDNA mutations in ageing has been questioned because the overall level of any of these mtDNA mutations in a tissue is usually considerably lower than the threshold level required to cause respiratory chain dysfunction. However, somatic mtDNA mutations associated with ageing are not evenly distributed but can accumulate clonally in certain cells where they cause a respiratory chain deficiency. This leads to a mosaic pattern with scattered respiratory chain-deficient cells in different tissues of aged individuals. The mitochondrial theory of ageing is supported by a wealth of correlative data as described above, but has remained highly controversial in the absence of supporting experimental data. We have now addressed this question by generating mtDNA-mutator mice by gene targeting of the gene for catalytic subunit of mitochondrial DNA polymerase. We will describe phenotypes in these mice and also present stategies for using these mice to generate mouse lines with specific mtDNA mutations.

O-21
Mitochondria-mediated nuclear mutator phenotype in *Saccharomyces cerevisiae*

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Using *Saccharomyces cerevisiae* as a model organism, we analyzed the consequences of disrupting mitochondrial function on mutagenesis of the nuclear genome. We measured the frequency of canavanine resistant colonies as a measure of nuclear mutator phenotype. Our data suggest that mitochondrial dysfunction leads to nuclear mutator phenotype (i) when oxidative phosphorylation is blocked in wild-type yeast at mitochondrial complex III by antimycin A and (ii) in mutant strains lacking the entire mitochondrial genome (rho0) or those with deleted mitochondrial DNA (rho $-$). The nuclear mutation frequencies obtained for antimycin A-treated cells as well as for rho $-$ and rho0 cells were $\sim 2-3$-fold higher compared to untreated control and wild-type cells, respectively. Blockage of oxidative phosphorylation by antimycin A treatment led to increased intracellular levels of ROS. In contrast, inactivation of mitochondrial activity

(rho − and rho0) led to decreased intracellular levels of ROS. We also demonstrate that in rho0 cells the REV1, REV3 or REV7 gene products, all implicated in error-prone translesion DNA synthesis (TLS), mediate mutagenesis in the nuclear genome. However, TLS was not involved in nuclear DNA mutagenesis caused by inhibition of mitochondrial function by antimycin A. Together, our data suggest that mitochondrial dysfunction is mutagenic and multiple pathways are involved in this nuclear mutator phenotype.

I-25
Treatment strategies for disorders of the mitochondrial genome

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Patients suffering from defects of the mitochondrial genome can present with a bewildering spectrum of clinical disorders. These chronic progressive diseases commonly affect post mitotic tissue and for the vast majority of patients there is no effective cure or treatment. In this overview, I will present several important and exciting bodies of work that have been reported by groups working in this demanding and frustrating area of research.

I-26
Use it or lose it?—Exercise training implications of mtDNA disorders

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To train or not to train, this is the question for patients with heteroplasmic mitochondrial DNA (mtDNA) disorders. Endurance exercise training induces adaptive proliferation of mitochondria in healthy muscle, increasing capacity for exercise and oxidative phosphorylation. Limited physical activity has the converse effect, with a down-regulation of mitochondria resulting in decreased exercise tolerance. In patients with mtDNA defects, we have consistently demonstrated benefits of endurance training that improve oxidative capacity. In a recent study, physiological and biochemical improvements were attributed to training-induced increases in mitochondrial numbers that in most patients resulted in increased levels of rate-limiting enzymes, suggesting increases in absolute levels of wild-type (wt) mtDNA. However, the concomitant finding of an increase in mutant to wt mtDNA ratio raised concern for advisability of endurance training. Furthermore, effects of longer-term training and cycles of physical inactivity (normally associated with a down-regulation of mitochondrial volume) following training (particularly in the setting of a new mutation load) on mutant and wt mitochondrial genomes are unknown. To determine whether training increases in mutant mtDNA levels may be offset by increases in absolute wt mtDNA levels and ultimately to determine advisability of endurance training as therapy, we have combined efforts with our collaborators in Newcastle. Preliminary results of this current study assessing the effects of training and detraining on physiology, biochemistry and single-fiber molecular genetics in heteroplasmic patients will be presented. Currently, we are unable to clearly advise our patients to ‘‘use it or lose it’’—underscoring the importance of these exercise training studies.

O-22
Aerobic training improves oxidative capacity in patients with mitochondrial myopathy

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Mitochondrial myopathies (MM) are characterized by exercise intolerance. We investigated the efficacy and safety of low-intensity dynamic exercise training in MM.

Seven patients with molecularly well-defined defects of mtDNA and seven healthy, age- and gender-matched subjects were studied. All subjects cycled for 30 min on a stationary bike, five times a week for 12 weeks, at an intensity corresponding to 65% of their maximal oxygen uptake. Maximal oxygen uptake, workload, heart rate, plasma lactate levels, and in patients, muscle mutation load were measured before and after training.

Aerobic training increased maximal oxygen uptake by $14 \pm 3\%$, workload by $28 \pm 12\%$ and the concentration of citrate synthase by $81 \pm 11\%$ and the activity of citrate synthase-corrected complex I activity by $101 \pm 16\%$ in muscle of MM patients, while the initial mutation load in muscle of $70 \pm 7\%$ was unaffected by training. Responses to aerobic training in the healthy control subjects closely paralleled those found in the MM patients. Training did not affect creatine kinase levels in MM patients, except temporarily in one.

This study shows that chronic aerobic training improves oxidative capacity in patients with MM. Since training did not induce an increase in plasma creatine kinase levels and muscle mutation load, aerobic exercise training seems to be a safe and effective method to improve endurance in MM.

Ongoing studies in patients with low mutation loads in muscle, in whom mutation load will also be studied after deconditioning following training, will more conclusively establish the safety and efficacy of training as a treatment in MM.

O-23
Ketogenic treatment reduces the proportion of mutated mitochondrial DNAs

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Large-scale partial deletions of mitochondrial DNA (mtDNA) cause sporadic Kearns–Sayre syndrome (KSS), a fatal multisystem disorder, in which the majority of mtDNAs in affected tissues is deleted. Since most mtDNA-related diseases, including KSS, are recessive, only a few wild-type mtDNAs (wt-mtDNAs) can compensate for the deleterious effects of many deleted mtDNAs. We have developed a pharmacological approach to treat KSS in vitro, in which we grow cells in medium containing ketone bodies replacing glucose as the carbon source. Cells containing 100% deleted mtDNA died after 5 days of treatment, whereas those containing 100% wt-mtDNA survived. Furthermore, in a cloned heteroplasmic cell line, the proportion of wt-mtDNA increased from 7% initially to ~ 23% after 5 days in ketogenic medium, and there was a dramatic improvement in mitochondrial protein synthesis in these cells, implying that they had crossed the threshold for respiratory chain function. We also provide strong evidence that treatment with ketone bodies caused this "heteroplasmic shifting" not only among cells (i.e. intercellular selection) but also within cells (i.e. intracellular selection).

Poster presentation

P-1
Addition of tenofovir to a didanosine-based highly active antiretroviral therapy increases mitochondrial toxicity

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Objectives: Tenofovir disoproxil fumarate (TDF) is a nucleotide analogue that has been reported to be free of adverse effects on mitochondria. We evaluate the effects of the introduction of TDF on a highly active antiretroviral therapy (HAART) schedule containing didanosine (ddI) on mitochondrial mass, mitochondrial DNA (mtDNA) content and cytochrome *c* oxidase (COX) activity.

Methods: Fifty HIV-infected patients receiving a ddI-based HAART schedule were recruited, and changed to ddI plus TDF (300 mg/day) and nevirapine (400 mg/day) ($n = 25$, cases) or maintained with the same HAART scheme ($n = 25$, controls). All patients were symptom-free with undetectable viral load along the study. Peripheral blood mononuclear cells (PBMCs) were obtained at 0 (baseline), 6 and 12 months. The quantity of mitochondria was assessed by the spectrophotometric measurement of the citrate synthase activity, the content of mtDNA by quantitative real-time PCR, and the activity of COX by spectrophotometry.

Results: Cases and controls maintained unchanged all mitochondrial parameters at 6 months with respect to baseline. Conversely, at 12 months we found that mtDNA content was reduced in both, cases and controls, while mitochondrial mass and COX activity were found to be significantly decreased only in cases.

Conclusions: A decrease in mitochondrial mass, mtDNA content and COX activity is detected after 12 months of the addition of TDF to HAART schedules containing ddI. This diffuse deterioration of mitochondrial parameters could be due to TDF itself, the increase of ddI concentrations caused by TDF, or both. The relevance of these biochemical findings in clinical practice remains to be determined.

P-2
Thymidine phosphorylase administration through platelets infusion transiently reduces nucleoside accumulation in MNGIE

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disease caused by mutations in the gene encoding the enzyme thymidine phosphorylase (TP). Lack of function of TP in MNGIE patients produces the toxic accumulation of thymidine (dThd) and deoxyuridine (dUrd), which affect mitochondrial functions. Thus, we predict that reduction of circulating levels of these nucleosides could be therapeutic. In healthy subjects, platelets contain abundant functional TP; therefore, administration of platelets to MNGIE patients is predicted to lower dThd and dUrd accumulation in blood, and possibly prevents mitochondrial dysfunction. In order to test the biochemical and clinical effects of platelets infusion on MNGIE, we treated a 23-year-old MNGIE woman with three infusions of 5×1011 platelets from a healthy donor every 3–4 days (period of treatment, 8 days). Infusion of platelets transiently provided circulating TP to the patient (30% to 40% of control values, 24 h after each infusion followed by a gradual decline of TP activity). Consistent reductions of dThd and dUrd plasma levels (to 50–70% of baseline values) were observed during TP restoration and, concomitantly, nucleoside urine excretion virtually disappeared. No clinical changes were detected by physical examination before and after the treatment period. Although clearly insufficient as a treatment, the partial reductions of dThd and dUrd after platelet infusion to MNGIE patients provide valuable data in support of an enzyme therapy-based strategy. Our results indicate that TP restoration in MNGIE patients can ameliorate biochemical imbalances that cause the disease.

P-3
Deoxyguanosine kinase (DGUOK) mutations in multiple mitochondrial respiratory chain deficiencies

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Deoxyguanosine kinase (DGUOK) is a mitochondrial protein involved in the supply of deoxyribonucleotides in mitochondria and therefore in the maintenance of the mitochondrial DNA. DGUOK mutations are associated with mitochondrial DNA depletion and result in multiple deficiencies of the respiratory chain. Complex II, encoded only by nuclear genes, is normal in those patients. Children with DGUOK mutations usually present a hepatocerebral disease. In order to estimate the prevalence of DGUOK mutations in multiple respiratory chain deficiencies (complex I+IV or complex I+III+IV), we have studied the DGUOK gene in a series of 32 patients with various clinical presentations and various tissue expression of the RC deficiency. We identified previously unreported DGUOK mutations in six patients. Three patients, born to consanguineous parents, were homozygous for a missense mutation (M1T and L250S in two sibs), one patient presented a homozygous 4-bp insertion (TGAT 796–799) and three patients were compound heterozygous (E165V/L226R and Q211G/L266R in two sibs). All these patients presented hypotonia and liver failure. Interestingly, some patients with hepatocerebral disease and multiple respiratory chain deficiency did not present DGUOK mutations.

These findings allowed us to propose prenatal diagnosis in two families. One pregnancy was interrupted but the second allowed the birth of a healthy child. In one family a neonatal diagnosis for the second sib permitted a prophylactic treatment with ursodeoxycholic acid. This child is actually 19 months old, has a normal neurological development and is on liver transplantation list.

In conclusion, a high prevalence of DGUOK mutations (22%) was observed in patients with multiple respiratory chain deficiencies. Moreover, patients with DGUOK mutations presented a homogeneous hepatocerebral phenotype as previously reported.

P-4
Exploring the use of the bacterial conjugation system to deliver DNA to mitochondria

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For reasons unknown, organellar DNA transfection methods that work in lower eukaryotes have failed to work in mammalian cells. The inability to transfect mitochondria with exogenous DNA is a great stumbling block to progress in making cell culture or mouse models of mitochondrial disease, and in understanding of mammalian mitochondrial genetics in general.

We are exploring the possibility of using components of the bacterial conjugation system as tools to deliver DNA to mitochondria. Specifically, we want to achieve the following goals:

(a) to show that DNA can be delivered to isolated mitochondria via the protein importation pathway in vitro using components of the bacterial conjugation machinery.
(b) to show that DNA can be delivered to mitochondria in vivo using the bacterial conjugation system.

In a later phase, we hope to be able to select for introduced mitochondrial genomes in mitochondria in living cells using metabolic and/or antibiotic selection strategies.

Some major hurdles in transfection of mitochondria in a genetically stable and heritable manner will be discussed.

P-5
MtDNA in myopathies: absence of a preferential haplotypic background for deletions and a search for inversions

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Pathogenic defects in mtDNA can be divided into point mutations or large-scale rearrangements. Single or multiple macrodeletions are found in patients with different myopathies, with symptoms including ocular and hearing problems, weakness, ataxia, and cardiac and endocrine disorders. These rearrangements have been associated with the presence of directly repeated sequences in its breakpoints, although the factors of its occurrence just in some of the regions and in some individuals have not yet been identified. Various studies have reported or denied connections between the phenotypic expression of mtDNA mutations and certain haplogroup(s). Namely, a relationship between haplogroups U/K and the clinical features of patients with single mtDNA common deletion was described. We analyzed patients ($n=31$) with single or multiple mtDNA deletions, and typed them for the HVRI and HVRII. We found no significant association between any of the most frequent haplogroups and the presence of the common deletion or with the development of other macrodeletions. Additionally, in 16 mtDNA deletions detected, 13 were not formerly reported, 7 of which present direct repeats of 2–12 bp on its breakpoints. We are now analyzing samples of patients with myopathies that do not exhibit mtDNA deletions or the substitutions at the positions 3243, 3271, 8344, 8356, 8993 and 9176. We are typing them for the only pathogenic mtDNA inversion

reported yet by a screening method of co-amplification using specific primers for the normal and the inverted sequences (in the region 3901–3908 bp). In 20 individuals already typed, the inversion was not detected.

P-6
Novel mutations in thymidine kinase-2 associated with fatal mitochondrial myopathy and mtDNA depletion

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Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are autosomal recessive disorders of infancy or childhood characterized by decreased mitochondrial DNA (mtDNA) copy number and respiratory chain dysfunction in affected tissues. The myopathic form of MDS has been reported to be associated with mutations in the thymidine kinase-2 (TK2) gene while the hepatocerebral forms have mutations in the deoxyguanosine kinase (DGUOK) gene. In this study we have analyzed three patients with MDS for mutations in the TK2 and DGUOK genes. One patient was compound heterozygous for two novel mutations in TK2: T33M in exon 3 and R152W in exon 8. The parents were heterozygous for the respective mutations showing that the mutations were allelic. The patient and one older sister died at 2 years of age due to rapidly progressive myopathy with respiratory failure. The diagnosis of mitochondrial myopathy with respiratory chain deficiency and hyperlactatemia was made at the age of 1 year. The muscle biopsy showed marked deficiency of cytochrome *c* oxidase in addition to muscle fibers with accumulation of ultrastructurally abnormal mitochondria. There was a severe reduction of mtDNA copy number (less than 10% of normal) in muscle tissue. Immunostaining showed severe reduction of mtDNA in cytochrome *c* oxidase-deficient muscle fibers with accumulation of mitochondria. Our findings extend the list of TK2 mutations associated with myopathic form of MDS.

P-7
Expression of ATPAF1 and ATPAF2 genes encoding F1-ATPase assembly proteins in mammalian tissues

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ATPAF1 and ATPAF2 genes code for specific assembly factors essential for biosynthesis of the catalytic F1 part of the mammalian ATPase. Nevertheless, their role in the control of ATPase biogenesis remains unclear. In one case of mitochondrial disease, the ATPase deficiency was ascribed to the ATPAF2 mutation. On the contrary, we were unable to find mutations in ATPAF2 or F1-subunit genes in several other nuclear defects of ATPase assembly. We examined levels of ATPAF1 and ATPAF2 transcripts in relation to the mRNA levels for F1-alpha and F1-beta, the interaction subunits, in mouse heart, kidney, brown fat, liver, muscle and brain by QT-RT-PCR. While ATPAF1 behaves as a uniformly transcribed housekeeping gene, ATPAF2 is highly regulated and its expression differs up to 30-fold in mammalian tissues, being the most active in ATPase-poor thermogenic brown fat. Although ATPAF2 mRNA levels are two orders of magnitude less abundant than F1-alpha and F1-beta mRNAs, the tissue-specific expression profile of ATPAF2 corresponds to that of F1-alpha and F1-beta genes. This is in accordance with the presence of several transcriptional elements, found to be active in various mitochondria-related nuclear genes, in ATPAF2 promoter region. Analysis of human fibroblasts showed about 10 times lower levels of ATPAF1 and ATPAF2 mRNAs compared to the transcripts of F1-subunits, and comparable levels of these transcripts in controls and patients with ATPase deficiency. Our results indicate that these ATPase defects are not caused by down-regulation of ATPAF1 and ATPAF2 genes.

P-8
MtDNA analysis in complex I-deficient patients

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Many patients are suspected to have a mitochondrial disease, based on their clinical symptoms or after biochemical investigations, although very often no genetic defect can be found. We selected a group of 20 patients with clinical and biochemical evidence of a decreased complex I activity, one of the most common enzyme defects of the OXPHOS disorders. The nucleotide sequence of mitochondrially encoded tRNA and MTATP6/8 genes had not revealed pathogenic mutations in these patients. The Denaturating Gradient Gel Electroforesis (DGGE) system was used to search for mutations in the seven mitochondrial-encoded subunits of complex I. In this group of 20 patients, a total of 96 nucleotide changes were

detected. Most of them were benign polymorphisms. Eleven sequence variations have never been described before, but since they did not fulfill any of the criteria for pathogenicity, we considered them as rather rare polymorphisms. Six of the 96 identified nucleotide variations were previously reported as pathogenic mutations, although a lot of controversy still exists about their pathogenic nature, especially because they are also found in some healthy controls. In six patients, up to eight or more polymorphisms were found. It has been suggested that it is the cumulative effect of these polymorphisms that causes disease. However, as we suspect that the 96 identified nucleotides changes are polymorphisms which cannot cause disease on their own, this study indirectly confirms the important role of the nuclear-encoded genes in causing complex I deficiencies.

P-9
MELAS and L-arginine therapy

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Background: Based on a hypothesis that the stroke-like episode in the syndrome of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is caused by impaired vasodilation in an intracerebral arterial segment, we evaluated effects of administering L-arginine, a nitric oxide precursor.

Methods: We assessed clinical status and biochemical parameters in venous blood during interictal and acute phases in 24 MELAS patients, and also in control subjects. In 34 stroke-like episodes, patients were infused with either 0.5 g/kg of L-arginine or a placebo. In some acute episodes, treatment effect on cerebral blood flow was studied; in some interictal patients, oral L-arginine prophylaxis was studied.

Results: Concentrations of L-arginine (46.6 + 12.7 μmol/l), citrulline (23.2 + 10.2 μmol/l), and nitric oxide metabolites (NOx; 24.0 + 9.8 μmol/l) during the acute phase of MELAS were significantly lower than in control subjects (108.1 + 27.6, 34.6 + 8.8, and 45.4 + 30.5 μmol/l, respectively), but asymmetrical dimethylarginine (ADMA) was not significantly decreased during this phase (0.45 + 0.10 μmol/l). At 30 min after L-arginine infusion, all symptoms suggesting stroke were significantly improved, in association with significantly increased L-arginine, citrulline, NOx, and cGMP. Increased tracer uptake showed improvement in blood flow in the freshly infracted region. Lactate and its ratio to pyruvate were restored to interictal-phase values 24 h after L-arginine infusion. With oral administration of L-arginine in the interictal phase, frequency and severity of stroke-like episodes decreased significantly.

Conclusions: L-arginine therapy showed promise in treating stroke-like episodes in MELAS.

P-10
Long-surviving Leigh syndrome patients with COX deficiency and SURF1 mutations

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Most study groups agree on early onset and death before adolescence to be major features of LS patients with COX deficiency and SURF1 mutations. We found two patients who survived their 10th birthday.

Patient 1 developed nearly normal in her first year. She then got retarded in psychomotor skills. A muscle biopsy revealed a COX deficiency. We sequenced the SURF1 exons and found compound heterozygosity for 574–575insCTGC and 74G > A, which leads to a frameshift and a nonsense codon, respectively. No Surf1 protein was detectable on Western blots. At age 18 she is mentally well preserved, though the disease clearly progresses.

Patient 2 developed symptoms in his second year. His psychomotor skills were well preserved for a long time, and he was able to attend for 1 year at a regular elementary school. He was diagnosed in his 7th year with elevated lactate levels in CSF and decreased COX activity in muscle. Molecular investigations in this patient detected a new mutation in SURF1 (269T>C) causing an exchange of leucine to proline. In Western blot studies we were able to detect a decreased amount of Surf1 protein. The boy is 13 years old now.

Almost all SURF1 mutations described so far resulted in complete loss of the protein and are associated with poor clinical prognosis. The long survival of two patients described here indicates that small amounts of Surf1 protein are protective against severe illness and that additional factors might compensate the devastating effects of complete protein loss.

P-11
Fungal strains with complex I mutations implicated in mitochondrial disease

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The primary function of mitochondria is to convert energy via oxidative phosphorylation, a process mediated by the mitochondrial respiratory chain. Mutations in mitochondrial proteins encoded by both mitochondrial and nuclear DNA are implicated in a wide range of mitochondrial diseases. Complex I deficiency is a major contributor to these disorders and mutations in nuclear-coded subunits of the enzyme have been specifically identified in severe pathological cases. We developed *Neurospora crassa* strains harbouring mutations in the TYKY, PSST and 51-kDa homologous subunits, equivalent to those found in human diseases. These subunits are involved in the catalytic activity of complex I and the point mutations target amino acid residues that are fully conserved between fungi and humans.

The point mutations P88L and R111H were separately introduced in the TYKY homologue, the V135M mutation was created in the PSST homologue and the A353V and T435M mutations were separately generated in the 51-kDa protein. All alterations were performed in cDNAs, which were then expressed in the corresponding null-mutant under the control of a heterologous promoter. P88L, R111H and V135M strains contain a fully assembled complex I in contrast to the respective null-mutants, where formation of the peripheral arm of the enzyme is prevented. Isolated complex I from these strains displays spectroscopic and enzymatic properties similar to those observed in the wild-type enzyme. Characterization of the A353V and T435M mutants in terms of enzyme structure and function is underway and will be presented.

P-12

Pathological findings of BAEP in Leber's hereditary optic neuropathy

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Introduction: Leber's Hereditary Optic Neuropathy (LHON) is a mitochondrial and multisystemic disease manifested by acute or subacute loss of central vision. Neurophysiological findings (electromyoneurography and multimodal evoked potentials) confirm asymptomatic involvement of the other organ systems. The aim of the study is to examine functionality of the auditory pathway using BAEP.

Materials and methods: Research included seven patients with clinically and genetically confirmed diagnosis of LHON, aged between 11 and 48 (average age 22.3 years), five males (71.4%) and two females (28.6%), and 13 asymptomatic family members from six families, aged between 12 and 53 (average age 30.1 years), four males (30.8%) and nine females (69.2%). All of examined subjects have BAEP done on the apparatus Medelec Sapphire Premiere.

Results: Three "primary" LHON mutations of the mitochondrial genome have been found on seven patients from seven families—three of them have 11 778, three have 3460 and one has 14 484. All of those seven have pathological BAEP with interpeak latency (IPL) prolongation between the first and the fifth wave (IPL I–V) and IPL I–III, unilaterally or bilaterally, while only one patient has IPL III–V prolongation on both sides. Examination of the family members showed IPL I–V prolongation in nine of them, among which five have IPL I–III prolongation, unilaterally or bilaterally, and only one unilateral IPL I–III prolongation. BAEP finding was normal on three family members. All examined subjects are previously tested and had normal audiometry.

Conclusion: Prolongation in I–III IPL suggests slowed conduction along eighth cranial nerve. Asymmetrically increased IPL III–V on the level of the brainstem is hard to explain. IPL I–V prolongation suggests conduction defect along the intraaxial acoustic pathways. Pathological BAEP indicates multisystemic character of the LHON.

P-13

Screening for somatic mtDNA alterations in neoplasias by denaturing high-performance liquid chromatography

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To elucidate the relationship between tumorigenesis and mtDNA alterations in various types of cancer, a rapid screening method for somatic mtDNA variations was established. Denaturing HPLC analysis was employed because it has been shown to have a high sensitivity and specificity for detection of mtDNA alterations. This is especially important in tumor tissues with low tumor cell content where somatic mtDNA mutations specific for the tumor cells might not be detected by conventional methods like sequencing. The entire mtDNA of tumor tissue

and the corresponding normal tissue from adult renal cell carcinoma (RCC) and childhood acute lymphoblastic leukaemia was amplified in 300–550-bp overlapping fragments. The DNA mixtures were denatured and reannealed, and the homo- and heteroduplices were evaluated by temperature-modulated heteroduplex analysis by DHPLC.

In RCC tissues ($n = 11$), we found two homo- and one heteroplasmic somatic mtDNA mutation. Independently of the presence of somatic mtDNA alterations, in all RCCs investigated, a dramatic decrease in the mtDNA content was detected. In leukemia samples ($n = 6$), we found two heteroplasmic somatic mutations. One patient showed more than 10 heteroplasmic mtDNA variations shortly after completion of chemotherapy. Further investigations revealed that the variations were not induced by chemotherapy but might be due to the transfusion of platelets.

Our screening method proofed to be able to detect somatic mtDNA mutations in a percentage which has already been reported for other tumors. In cases of allogeneic platelet infusion, care has to be taken with interpretation of mtDNA sequence variations in blood samples of leukemic patients.

P-14
Autosomal recessive mitochondrial ataxic syndrome due to mitochondrial polymerase gamma mutations

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We describe three families and one sporadic case with a syndrome that includes a progressive ataxia, epilepsy, myoclonus, tremor, peripheral neuropathy, headache and ophthalmoplegia. In most cases, headache and/or epilepsy is the first symptom and ophthalmoplegia is a late feature. The ataxia is due to a combination of central and peripheral disease and the commonest form of epilepsy was focal and involving the occipital lobes. MRI shows changes in the central cerebellum, olivary nuclei, occipital cortex and thalami. Mitochondrial dysfunction is suggested by the finding of cytochrome *c* oxidase (COX)-deficient fibres in muscle, although in one case these were rare and in another completely absent. Multiple mitochondrial DNA deletions could be demonstrated in all affected individuals. Deletions could be detected using standard methods of analysis in two families, but in two cases, including the one with no COX-deficient fibres, real-time PCR analysis was required to confirm the presence of this abnormality. Genetic studies reveal mutations in the mitochondrial DNA-dependent DNA polymerase gamma (POLG) gene. These findings confirm the importance of POLG in the causation of recessive neurological disease, show that mutations in this gene cause a much wider phenotype than recognised previously, and demonstrate how the phenotype may evolve with time. The study also highlights the importance of using sensitive methods for detecting the presence of multiple mtDNA deletions.

P-15
Mitochondrial therapies for HIV lipoatrophy

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The goal of this study is to identify interventions to reverse or delay the progression of lipoatrophy occurring in HIV-infected individuals following treatment with antiretroviral therapy. The combination of mitochondrial co-factors, co-enzyme Q10 and acetyl-L-carnitine, together with Niaspan and Pioglitazone are being evaluated in a 24-week pilot investigation of HIV lipoatrophic patients with two study arms. There is a treatment group ($n = 10$) and a control group ($n = 10$) that will undergo the same assessments. Patients are being monitored for changes in fat content by whole-body dual energy X-ray absorptiometry (DEXA) and abdominal eight-slice CT scan. Serial fasting lipids, glucose, insulin, and lactate levels will also be performed. To date, three male patients on the treatment arm and one male and one female patient on the control arm have completed the study. The DEXA analyses of arms, legs, trunks, and muscle peripheral fat did not change in either group. More interestingly, lactic acid levels decreased by 24% over the 24 weeks in the treatment arm (from an average of 2.1 to 1.6 mmol/l), whereas in the control arm, lactic acid levels increased by 36% (from an average of 0.9 to 1.4 mmol/l). In conclusion, the preliminary clinical metabolic data suggest that this intervention may be affecting mitochondrial metabolism.

P-16
***Drosophila* VDAC: a model system to study mitochondrial function**

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Voltage-dependent anion channels (VDACs) are a family of small pore-forming proteins of the mitochondrial outer membrane found in all eukaryotes. VDACs play an important role in the regulated flux of metabolites between the cytosolic and mitochondrial compartments and three distinct mammalian isoforms have been identified. The specific physiologic and potential pathophysiologic roles of the various isoforms are not understood, but animal and cell culture experiments suggest that the various isoforms function in apoptosis, learning and reproduction. Recent annotation of the *Drosophila melanogaster* genome has revealed three putative VDAC isoforms (CG17137, CG17139, CG17140) closely linked to the previously reported *Drosophila* VDAC (porin). Molecular characterizations reveal a complex sex-specific expression pattern for these genes. When expressed in yeast deficient for VDAC, porin and CG17137 rescue a conditional-lethal phenotype. Electrophysiological characterization of these proteins in reconstituted systems demonstrates a differential ability to permeabilize lipid bilayers and liposomes. Analysis of flies homozygous for hypomorphic P element alleles of porin reveals abnormal phenotypes including partial lethality, neuromuscular dysfunction and male infertility phenotypes reminiscent of mouse VDAC mutants. Transgenic expression of porin in the homozygous, hypomorphic background rescues the partial lethality and neuromuscular phenotypes. Tissue-specific overexpression of CG17139 and CG17140 leads to developmental defects, while a truncating deletion of CG17140 results in recessive lethality. These studies demonstrate that *Drosophila* VDAC homologs have retained important aspects of VDAC structure and function, warranting further studies to utilize this genetic model system in delineating fundamental conserved roles of VDACs and mitochondria in eukaryotic cell function.

P-17
Mitochondrial gene expression and transcription in Alzheimer's disease patients

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We present an analysis of the steady-state levels of mitochondrial RNA and an estimation of the transcription rates in cerebellum, hippocampus, frontal cortex and lymphocytes from AD patients and controls. The expression of the genes ND4, 12S, Cyt b, COX II and ND6 (encoded by the mtDNA) in AD patients and controls has been measured by performing reverse transcription followed by real-time PCR quantification method based on Lightcycler technology using SYBR Green I dye. Gene expression was also analysed by performing classic dot blot experiments to compare the two techniques. Estimation of the transcription rate was calculated by combining the mtDNA data and RNA data obtained for the gene expression studies. Our real-time PCR experiments of gene expression revealed results equivalent to those obtained with dot blot experiments. Real-time PCR could be the technique of choice to replace classic laborious, time- and sample-consuming experiments such as Northern, dot or slot blots for analysing gene expression. The relationship between mtDNA content, steady-state RNA levels and transcription rates in these AD samples is also discussed.

P-18
Generation and characterization of a new loxable knockout model of the Surf1 gene

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Leigh encephalopathy associated with COX deficiency is one of the most common disorders of the mitochondrial respiratory chain in infancy. In most cases it is due to loss of function mutations of Surf1, a COX assembler. We have developed a first constitutive knockout (KO) mouse model for Surf1 by replacing the central region of the gene with a Neo cassette (Neo allele). The Surf1 null Neo/Neo mice were characterized by the following features: (1) high post-implantation embryonic lethality, affecting approximately 90% of the $-/-$ individuals; (2) significant deficit in muscle strength and motor performance; (3) a partial, isolated defect of COX activity in skeletal muscle and liver, and, to a lesser extent, heart and brain; (4) absence of overt neurological symptoms. To test whether the high embryolethality in Neo/Neo individuals was due to a role of Surf1 in early embryogenesis, we created a second murine model characterized by a minimal structural perturbation of the recombinant alleles. This was achieved by introducing a Neo cassette flanked by two lox-P sites (neolox-P allele) within exon 7 of the Surf1 gene. The neolox-P/+heterozygous animals were normal but never gave birth to homozygous neolox-P/neolox-P individuals. By contrast, after elimination of the Neo cassette by using the cre recombinase, the percentage of homozygous lox-P/lox-P KO animals was restored to the expected 25%. The animals showed high level of lactic acid in the blood and a generalized COX defect. The rotation-rod test was normal and there was no spontaneous neurodegeneration. These results demonstrate that (i) the absence of Surf-1 does not compromise per se embryonic vitality, and (ii) unknown compensatory mechanisms prevent the Surf1 null lox-P/lox-P mice from developing the severe phenotype observed in humans.

P-19
Differential display analysis of thyroid oncocytoma: searching for mitochondrial proliferation mechanism

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In order to find new proteins involved in the mitochondrial biogenesis and/or in oncocytoma tumorigenesis, we performed a differential display analysis on two thyroid oncocytic adenomas, tumours characterised by the presence of a large number of mitochondria, versus paired control thyroid tissue. The 360 overexpressed and 100 underexpressed fragments in the tumour were spotted on Nylon membranes macro-arrays with additional mitochondrial genes. The hybridisation of four other paired oncocytoma/normal thyroid couples on the macroarrays confirmed the differential expression for 49 overexpressed and 12 underexpressed fragments. Among the overexpressed genes, genes coding for mitochondrial components are well represented with 12 mtDNA-encoded genes and two nuclear-encoded genes, UCP2 and ANT2, of which up-regulation had already been described in oncocytoma. The Cathepsin B gene is involved in the processing of the thyroglobulin and has been shown to be up-regulated in human thyroid tumours. The remaining known genes are involved in various metabolisms (transcription modulation, translation, ubiquitination, etc.). Among the underexpressed genes, the Pendrin, which codes for an apical transporter of chloride/iodide ions, is known to be modulated in expression and location in a context of thyroid tumour. In silico, five unidentified genes show, in their promoter, potential regulatory sequence for transcription factors known to be involved in the mitochondrial biogenesis; for three of them, the potential corresponding protein shows a putative N-terminal mitochondrial targeting signal. Further analysis to investigate the involvement of these genes in mitochondrial biogenesis and/or thyroid tumorigenesis is ongoing.

P-20
Investigation of mitochondrial function in the pilocarpine model of epilepsy

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The presence of epileptic seizures as one of the manifestations of several mitochondrial disorders may suggest the involvement of mitochondrial dysfunction in epileptogenesis. Considering the important role of mitochondria on neuronal cell death and generation of reactive oxygen species (ROS), the findings of apoptosis and increased production of ROS in experimental induced epilepsy support this hypothesis. However, studies investigating mitochondrial involvement in epilepsy showed variable results: deficiency in complex I or IV (in patients), deficiency in complex I and IV (in pilocarpine model), increased expression in subunit I of cytochrome *c* oxidase (COX) and complex I (in genetic model) and normal COX activity (in kindling model).

We investigated the possible role of mitochondrial dysfunction in epileptogenesis using the pilocarpine model of temporal lobe epilepsy. We studied mtDNA abnormalities (by PCR and Southern blotting), COX expression and activity (by Western blotting and immunohistochemistry) in rats in the chronic phase of the model, when spontaneous and recurrent seizures begin to occur.

A 4.8-kb mtDNA deletion was detected by PCR but with a frequency similar to the age-matched control group. No other rearrangements or quantitative alterations of mtDNA were detected. Abnormalities of mitochondrial protein (COX I, COX IV, SDH) expression were not detected in hippocampus by Western blotting or immunohistochemistry. COX activity (by histochemistry) did not show abnormalities in different regions of hippocampus in experimental animals. In conclusion, our results could not demonstrate a mitochondrial involvement in epileptogenesis. (Supported by FAPESP).

P-21
Renal oncocytomas as a model to study the regulation of mitochondrial proliferation

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Mitochondrial defects have been associated with severe neurodegenerative disorders and with human cancers. A variety of encephalomyopathies have been linked to alterations of mtDNA. The predominantly neurological symptoms are not produced by the mutations per se, but by an unregulated proliferation of pathologic mitochondria. We are using oncocytomas, which are mostly benign epithelial tumors whose predominant feature is a massive accumulation of mitochondria in the cytoplasm, as a way to search for unknown genes that play a role in regulation of mitochondrial proliferation.

We studied three oncocytomas with 11q13 rearrangements. Fluorescent in situ hybridization (FISH) using BAC

clones in the region showed that the 11q13 breakpoints in all three tumors are near the CCND1 (BCL1) gene. None of the breakpoints disrupts any known gene other than CCND1, but gene prediction programs suggest several human genes in the breakpoint region. The rearrangement in one tumor consisted of a segmental duplication that included 11q13, presenting several mitochondrially targeted genes immediately distal to CCND1 as interesting candidate genes.

P-22

Role of the ND4L and the ND9 subunits in the activity and assembly of complex I in *Chlamydomonas reinhardtii*

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Seven to nine subunits (ND1–7, 9, 4L) are usually encoded in the mitochondrial genomes of eukaryotic cells. In *Chlamydomonas reinhardtii*, only five subunits (ND1, ND2, ND4, ND5 and ND6) are mitochondrial-encoded. Genomic analysis has allowed us to identify the Nd3, Nd4L, Nd7 and Nd9 genes in the nuclear genome of this alga. In this study, the Nd4L and Nd9 genes have been inactivated by RNA interference. For that purpose, constructions bearing a fragment of the targeted genes in the sense direction followed by the corresponding cDNAs in the antisense direction have been put under the NIT/ARS promoter of *Chlamydomonas reinhardtii*. Transformant cells with an intact copy of the chimeric construction have been identified after cotransformation. Activity and assembly of complex I have been analysed in the transformed cells. Northern blots have been performed in order to detect cells showing a reduced or a null expression of the Nd4L or Nd9 genes. Our results showed that in cells where the expression of Nd4L or Nd9 has been suppressed, activity and assembly of whole complex I are abolished.

P-23

Modifiers of phenotype in a *Drosophila* model of mitochondrial disease

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Mutations in the maternally inherited mitochondrial genome (mtDNA) or in nuclear genes involved in either mitochondrial metabolism or mtDNA maintenance manifest with a wide range of clinical phenotypes, commonly affecting 'high energy' tissues (heart, skeletal muscle, the CNS and sensorineural epithelia).

Our work is focused on exploiting a *Drosophila* mutant in the nuclear gene technical knockout (tko), encoding mitoribosomal protein S12, which is involved in fidelity of protein synthesis. One mutation, tko25t, giving the substitution L85H in a conserved amino acid of the protein, confers a phenotype with some resemblances to human mitochondrial diseases, such as developmental delay, paralytic seizures, hyporeactivity and defective auditory function.

Continuous selection during inbreeding of tko25t mutant flies in an initially mixed genetic background produced different sub-lines showing various degrees of recovery towards the wild-type phenotype. These should represent useful paradigms for identifying genes and pathways that can or do modify phenotype in human mitochondrial disease. We present a preliminary transcriptomic analysis of these lines, identifying modifier genes responsible for the restoration the phenotype in these lines.

P-24

Relationship between mutational load and increased lactate production in olfactory epithelial cells with the MELAS a3243g mutation

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Background: MELAS syndrome is associated with the MELAS A3243G mutation. In its most severe form, affected patients suffer stroke-like episodes; focal neurological deficits associated with lactic acidosis and a clinical prodrome of headache, nausea or vomiting. Lactic acidosis often heralds the onset of a stroke-like episode.

Aim: To determine the relationship between lactic acidosis and mutational load in neural cell lines containing the MELAS A3243G mutation.

Methods: Olfactory epithelial biopsies were obtained from a patient with MELAS A3243G and 7 control subjects. Cultured cells were stained for glial markers that indicated a neural origin. Lactic acid production was measured after cultured cells that had been deprived of glucose for 1 h. To reduce mutational load, cells were cultured in galactose-supplemented (glucose-free) media for 2–6 days. Mutational load was determined using PCR/RFLP analysis.

Results: Cells cultured from olfactory epithelial biopsies were shown to express GFAP, indicating that they were neural in origin. Cells with the MELAS A3243G mutation produced an increased amount of lactate (3μmol/h/mg protein) when compared to cells derived from control subjects (1.8μmol/h/mg protein). Mutational load of MELAS A3243G containing cells decreased from 56% to

4% after culturing in galactose-supplemented (glucose-free) media for 2 days. Lactic acid accumulation returned to normal control values when the mutational load decreased.

Conclusions: Cells harbouring the pathogenic MELAS A3243G mutation produce more lactate than cells derived from control subjects. A decrease in mutational load significantly reduced the lactate production of these cells. High mutational loads are responsible for the increased lactate production in neural cells containing the MELAS A3243G mutation.

P-25

An anti-apoptotic viral protein that recruits Bax to mitochondria

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The viral mitochondria-localized inhibitor of apoptosis (vMIA), encoded by the UL37 gene of human cytomegalovirus, inhibits apoptosis-associated mitochondrial membrane permeabilization (MMP) by a mechanism different from that of Bcl-2. Here we show that vMIA induces several changes in Bax that resemble those found in apoptotic cells, yet take place in unstimulated, non-apoptotic vMIA-expressing cells. These changes include the constitutive localization of Bax at mitochondria where it associates tightly with the mitochondrial membrane, forming high molecular weight aggregates that contain vMIA. vMIA recruits Bax to mitochondria, but delays relocation of caspase-8-activated truncated Bid-GFP (t-Bid-GFP) to mitochondria. The ability of vMIA and its deletion mutants to associate with Bax and to induce relocation of Bax to mitochondria correlate with their anti-apoptotic activity and with their ability to suppress MMP. Taken together, our data indicate that vMIA blocks apoptosis via its interaction with Bax. vMIA neutralizes Bax by recruiting it to mitochondria and ''freezing'' its pro-apoptotic activity, yet undescribed viral strategy of subverting an intrinsic pathway of apoptotic signaling.

P-26

CDNA-based screening and analysis of the OPA1 gene in patients with autosomal dominant optic atrophy

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Autosomal dominant optic atrophy (adOA) is the most common form of inherited optic neuropathy. The predominant locus was linked to chromosome 3q28–q29 and the disease-causing gene OPA1, which encodes a dynamin-related GTPase targeted into mitochondria, was identified. Up to now, 89 different OPA1 mutations in 159 unrelated families have been identified.

Because of the complex structure of the OPA1 gene within 30 coding exons, the commonly used mutation detection technique, exon by exon sequencing, is laborious and expensive. Moreover, putative splice site mutations cannot be functionally characterised and mutations in the unspecified promoter region or larger deletions may not be detected. To overcome these problems, we establish a cDNA-based screening analysis using RNA isolated from blood leukocytes or cell lines. RNA samples were reverse transcribed, the entire coding region of the OPA1 gene was amplified as four overlapping cDNA fragments and screened by direct sequencing. Using this method we have identified 34 different OPA1 mutations including 6 frameshift, 10 missense, 3 nonsense and 15 splicing mutations. Twelve of these splicing mutations lead to skipping of the flanking exon and three of them to an activation of a cryptic splice site. We noticed that some mutations that lead to a premature stop codon are underrepresented at the cDNA level, which most likely results from nonsense mediated mRNA decay. Allele-specific cDNA quantification applying pyrosequencing was used to confirm and quantify the reduced levels of the mutant transcripts. These results provide additional evidence that haploinsufficiency may represent a mechanism for the development of adOA.

P-27

Expression analysis of the dynamin related OPA1 protein in the human brain

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Autosomal dominant optic atrophy (adOA) is the most prevalent hereditary optic neuropathy. The majority of cases of adOA is associated with mutations in the OPA1 gene. This OPA1 gene encodes a 1015-amino-acid polypeptide (approximately 110 kDa) with similarities to GTP-binding proteins of the dynamin protein family. The OPA1 protein is imported into mitochondria where it is attached to the inner membrane facing the intermembrane space. OPA1 may represent the human ortholog of the *S. cerevisiae* MgmI and the *S. pombe* MspI proteins, which are important for mitochondrial inheritance and maintenance in

these organisms. Northern blot analyses show that OPA1 is expressed in all tissues examined, with the highest transcript levels in the retina and in the brain. Here we addressed the cell type specific expression of the OPA1 protein in human brain sections using immunohistochemical techniques and Western blot analysis.

We studied OPA1 expression in normal brains obtained at autopsy from patients with no reported neurological symptoms or diseases. We analysed paraffin sections and tissue homogenates of the cerebellum and different areas of the cerebrum using a polyclonal antibody raised against a carboxy-terminal peptide of OPA1.

We found OPA1 expression in somata and dendrites of neurons of the layers II–VI of the motor cortex and frontal lobe. In the cerebellar cortex OPA1 expression was detected in the Purkinje cell layer, in the granule cell layer and in the molecular layer. Double labelling experiments showed that OPA1 is also expressed in GFAP-positive astrocytes, which was confirmed by Western blot analysis.

P-28
Two families with milder course of Leigh syndrome associated with SURF1 gene mutations

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COX-deficient Leigh syndrome (LSCOX –), inherited as an autosomal recessive trait, is one of the most common encephalomyelopathies of infancy or childhood. Clinical presentation includes muscle hypotonia, developmental delay, psychomotor retardation, respiratory abnormalities and brainstem or basal ganglia dysfunction. The elevated lactate level in cerebrospinal fluid and serum is observed. The pathological hallmarks of LS are bilateral, symmetric necrotizing lesions in the basal ganglia, thalamus, brainstem and spinal cord. It was established that mutations in the SURF1 gene are responsible for the most cases of Leigh syndrome associated with severe COX deficiency. The SURF1 gene, located on chromosome 9q34, is engaged in assembly of cytochrome *c* oxidase complex. Most patients carry frameshift or nonsense mutations predicting loss of function of the SURF1 protein. Missense mutations are uncommon, as only a few have been described in typical COX-deficient Leigh patients.

We present two families (seven children) with mild clinical course of the disease. One of two novel missense mutations, M235T or Y274C, in combination with recurrent frameshift 845delCT was identified in each patient. Enzymatic and histopathological study of muscle biopsy specimens revealed decreased COX activity and normal/increased citric synthase values. Progression of the disease is relatively slow. Three of the patients survived 18, 17 and 12 years with undisturbed mental development. They demonstrate only light respiratory involvement. Our patients belong to a small group of a few reported cases with the phenotype of late Leigh syndrome associated with SURF1 mutations.

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P-29
Mitochondrial disease: new prevalence figures with major resource implications

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The true prevalence of mitochondrial disease within Europe is uncertain. Increased clinical awareness and advances in molecular genetic techniques have led to increasing numbers of diagnoses in most specialist centres, yet mitochondrial disease is still considered to be a rare condition, attracting little in the way of specific resource allocation. Marked clinical heterogeneity, diverse referral patterns, and asymptomatic carriage of mitochondrial DNA mutations undermine attempts to accurately measure its prevalence.

The Newcastle Mitochondrial Centre acts as a tertiary referral centre for mitochondrial disease in the North East of England. Within genetically characterised pedigrees, we identified clinically affected adults below retirement age, living within the North East at the midpoint of 2001. Pedigree analysis was then performed to identify relatives directly at risk of developing mitochondrial disease. The minimum point prevalence of clinically affected adults in the North East of England was 10/100,000. The prevalence of those affected or at risk was 28.6/100,000. This equivocates to nearly 17,000 individuals within the UK and over 230,000 individuals throughout Europe. These figures are far in excess of previous estimates and do not include large paediatric cohorts.

The diagnostic processes, family tracing, and associated genetic counselling involved in confirming these figures merely represent our basic clinical obligation in a progressive and disabling genetic disease. At present there are few guidelines for the genetic and clinical diagnosis, or the management of these important disorders. Our study highlights the pressing need for such guidelines, especially in view of the complex genetics in many patients.

P-30
Mitochondrial DNA variation and Parkinson disease

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Mitochondrial dysfunction has been implicated in the pathogenesis of Parkinson disease (PD). Complex I defects have been documented in the substantia nigra and platelets of patients with PD. Cybrid studies have implicated mitochondrial DNA (mtDNA) but the underlying gene defects have yet to be identified. In a recent North American study, mtDNA haplogroup J and haplogroup K were associated with a decreased risk of PD when compared to mtDNA haplogroup H. Here we report our study of mtDNA haplogroups in two large independent European cohorts of 515 patients with PD and 292 controls subjects.

P-31
Mitochondrial toxicity: mtDNA depletion in peripheral blood mononuclear cells from HIV-infected individuals is enhanced after co-infection with HBV/HCV

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Mitochondrial damage seems to be responsible for many of the long-term toxicities associated with treatment of HIV infection with nucleoside reverse transcriptase inhibitors. However, its incidence, and the precise role of different compounds and of HIV infection itself have not been elucidated yet.

To evaluate possible factors associated with mitochondrial damage in HIV infection, mitochondrial DNA (mtDNA) was measured in PBMCs from a total of 69 individuals. A duplex real-time NASBA assay was used and experiments were run in duplicate. The mtDNA copy number per cell was estimated based on the parallel measurement of nuclear DNA in one-tube reaction for each individual. Serum lactate, lipids, hepatitis B and C status and current antiretroviral therapy were recorded.

The median mtDNA value for the 11 HIV-negative persons was 773 copies/cell (IQR: 587–921), whereas it was 352 copies/cell (IQR: 257–529) among 58 HIV-positive patients ($P < 0.001$). Among HIV-pos individuals, 48 on antiretrovirals tended to show median mtDNA values higher than 10 subjects who were drug-naive (353 versus 294 copies/cell; $P = 0.320$). Overall, lower mtDNA levels were associated with higher lactate levels. In HIV-pos patients, lactate and mtDNA were significantly correlated ($P = 0.025$). Finally, co-infection with HCV and/or HBV was significantly associated with lower mtDNA values ($P = 0.016$). We conclude that HIV infection reduces mtDNA in PBMCs. Antiretroviral therapy tends to alleviate this depletion. There is a correlation between lactate levels and mtDNA. Interestingly, chronic infection with hepatitis viruses could result in further loss of mtDNA in HIV-positive patients.

P-32
The pathophysiological mechanism of thymidine kinase 2 deficiency in mtDNA depletion

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The thymidine kinase 2 (TK2) is a key enzyme in the mitochondrial nucleotide salvage pathway, providing the mitochondria with deoxyribonucleotides essential for mitochondrial DNA (mtDNA) synthesis.

Mutated TK2 is associated with mtDNA depletion and respiratory chain dysfunction, manifesting as severe skeletal myopathy in infancy.

Although the diagnosis of TK2 deficiency has been facilitated by the finding of several new mutations, the pathophysiological mechanisms of this defect remain to be elucidated. The reasons for normal fetal development, the sparing of non-affected tissues and the events leading to decreased mtDNA synthesis are still obscure.

We speculate that normal fetal development is maintained by the activity of cytosolic nucleoside kinases. Our findings indicate that residual TK2 activity is responsible for the lack of involvement of certain tissues. Similarly, we show that imbalanced mitochondrial dNTP pools with markedly decreased thymidine triphosphate content are the major aberrations in fibroblasts of TK2-deficient patients.

P-33
Stimulation of oxidative phosphorylation complex biogenesis by the von Hippel Lindau factor

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According to the Warburg's hypothesis, mitochondrial function is impaired in several types of cancer. In agreement with this hypothesis, we had previously shown that, in renal carcinomas, low oxidative phosphorylation correlates with tumor aggressiveness. The mechanisms underlying this impairment are not understood. In the most aggressive renal cancers, the clear cell renal carcinomas (CCRCs), the tumor suppressor gene VHL (von Hippel Lindau factor) is invalidated. Hence, the transfection of the VHL gene in cells depleted of VHL suppresses their ability to form tumors when they are injected into nude mice. However, several genetic alterations are observed in addition to the VHL inactivation. In order to know whether VHL invalidation was responsible for the mitochondrial impairment, we have compared mitochondrial functions in 786-0 or RCC10 cells originally deficient in VHL to those of the same cells transfected with ectopic VHL. We show that the presence of pVHL increased mitochondrial contents of respiratory chain complexes and restored their functions. The presence of VHL permitted the cells to rely on their mitochondrial ATP production when growing in the absence of glucose. In addition, we show that the VHL-dependent activation of the OXPHOS complex biogenesis was not associated to significant changes in transcript amounts of nuclear factors: mitochondrial transcription factor A, Tfam, or other redox-responsive nuclear transcription factors such as NRF-1 or NRF-2. Therefore, another VHL-dependent regulatory mechanism of OXPHOS biogenesis is postulated.

P-34
Analysis of the subunit composition of mitochondrial respiratory complexes from different species

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We have used Blue Native Polyacrylamide Gel Electrophoresis (BN PAGE) to separate the mitochondrial respiratory complexes from different species.

The presence and composition of the five respiratory complexes were analyzed by combining three different methods:

– histochemical staining methods to detect the enzymatic activities of complexes I, II, IV and V directly in blue native gels [Electrophoresis 18 (1997) 2059–2064]
– immunodetection of subunits with specific antibodies
– mass spectrophotometry to determine the subunit composition of the complexes.

In the latter method, the BN PAGE band was isolated and fragmented by enzymes or chemical reagents. The peptides generated were then analyzed by LC-MS/MS.

These methods were used to determine the effect of various mutations on the assembly of the respiratory complexes, in particular for different oxa1 mutations in *Saccharomyces cerevisiae* and *Podospora anserina*.

P-35
Direct repeats in mtDNA constrain life span in mammals

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Deletions are often found in the mtDNA of elderly organisms. These deletions are often, though not always, flanked by direct repeats. From an analysis of the mtDNA sequences of 61 mammalian species we find that there is a constraint relationship between the number of the longer repeats (>11 bp) and life expectancy for each species. We also show that for most species the number of repeats in the mtDNA sequences is greater than the number in the corresponding randomly shuffled sequence, with the largest increases occurring among the short lived species. We will discuss other related factors, such as repeat location within the genome, repeat length and base composition.

P-36
Leigh's Syndrome in siblings carrying the Focal Bilateral Striatal Necrosis 9176T>C mutation

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A male infant of Greek and British ancestry presented at 9 months of age with a sudden loss of developmental milestones. An MRI scan revealed bilateral altered signals in the caudate nuclei, putamina and globus pallidus consistent with Leigh's syndrome. Since this episode he has made slow motor progress and at 30 months has central hypotonia, mild dystonia of his arms but no speech or swallowing difficulties. He walks with an ataxic gait and is intellectually normal. His younger sister had normal development until 7 months after which she regressed over a 3-month period. She subsequently regained the ability to smile and interact but at 15 months had an acute presentation with hypotonia, encephalopathy and tachypnoeia. She was found to have a plasma lactate of 5.3 mmol/l and a renal tubular acidosis and was treated with oral bicarbonate; all developmental milestones were lost. Since then she has made slow and steady

developmental progress. At 20 months she has central hypotonia, a well-developed pincer grasp, limb ataxia, drooling and some intellectual impairment but no dystonia. Overall she is more severely affected than her brother. Blood mitochondrial DNA analysis by a PCR/RFLP method and by sequencing revealed apparent homoplasmy for the Focal Bilateral Striatal Necrosis (FBSN) 9176T>C mutation in both siblings and a level of approximately 50% in the asymptomatic mother. This mutation affects subunit 6 of the ATP synthetase complex and has been reported to date in nine families with Leigh-like syndromes showing varying severity including severe, slowly progressive and non-progressive phenotypes.

P-37
Two regions with multiple heteroplasmic length variants in the mitochondrial DNA of a family with diabetes and hearing disturbances

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We have identified two locations (nt568 and nt8281) with multiple heteroplasmic length variants in the mitochondrial DNA of a family originating from Asia Minor. The nt8281 length variants stably inherit in a maternal way with no major changes in the length distribution in leucocytes of the offspring. In contrast, during culture of the fibroblasts derived from the proband, the length of the homopolymeric cytidine tracts is increased with on average two additional insertions. Furthermore, the heterogeneity in the polycytidine tract starting at position 568 is also extended by on average one cytidine upon culture of these fibroblasts.

Therefore, a mechanism seems active that drives these fibroblasts towards the enrichment of longer cytidine tracts. Functional analysis revealed that aberrations in the intergenic COII-tRNALys and the 568 regions are insufficient to induce a detectable decrease in mitochondrial respiration.

P-38
Functions and dysfunctions of the human dynamin OPA1

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The OPA1 mitochondrial dynamin, localised to the IMS associated to the cristae membrane, is involved in an inherited neurological retinopathy. Extinction of OPA1 expression in Hela cells induces major mitochondrial modifications including fission of the network, loss of the DeltaPsi, cristae destructuration and ultimately apoptosis, suggesting a pleiotropic function of OPA1.

We will present data concerning the singular functions of the different OPA1 variants and the effects of expressing pathological OPA1 alleles on the structure and dynamics of mitochondria and on the apoptotic process.

P-39
Lethal MELAS syndrome in a teenager with mtDNA tRNA 3271 mutation

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MELAS typically presents as stroke-like episodes leading to focal neurological deficits, seizures, headache and cognitive deterioration. An A-to-G transition at mt (t)RNA 3243 is present in 80% of the patients. Another tRNA mutation, T3271C, is found in about 10% of MELAS patients, mostly of oriental origin. According to Tarnopolsky et al. (1998), the two phenotypes are similar, but the age of onset is later in T3271C mutation. Our patient was the first of three children, with healthy parents and two sisters. Her early growth and development were normal. She was investigated at the age of 6 because of weight loss and poor growth. Exercise intolerance, WPW syndrome and cystic changes in the thyroid gland led to the search of a mitochondrial disease. Her muscle showed RRFs and complex I deficiency. No mtDNA arrangements were discovered. At the age of 14 strenuous exercise led to severe lactic acidosis, kidney failure, and pericardial and pleural effusion. Frequent partial seizures without loss of consciousness began at 17 years, and she died within 2 months after rapid increase in seizure frequency. Previously normal MRI showed now stroke-like lesions. Neuropathological changes were compatible with MELAS. The T3271C mutation was discovered post mortem. The proportion of T3271C mutant was over 90% of total mtDNA in her fibroblasts, and the amount of mutant mtDNA was 78–96% in other tissues. The amount of mutant mtDNA in blood leucocytes of mother and two younger sisters was 5%, 27% and 4%, respectively. The sister with 27% mutant mtDNA had developed diabetes at the age of 18. Hospital for children and adolescents 1, Department

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P-40
Respiratory chain enzyme deficiency expressed during muscle development

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We studied myoblast cell cultures from four unrelated, paediatric patients with marked mitochondrial respiratory chain enzyme deficiencies in skeletal muscle, but with no apparent mitochondrial DNA abnormalities. Despite the obvious enzyme defects in muscle tissue, biochemical assays revealed that respiratory chain enzyme activities were normal or only mildly affected in the myoblast cultures. Immunoblot analysis of native gels and cytochemical staining of the myoblast cultures corroborated the results of the biochemical assays. Myotubes were, on the other hand, clearly affected in all four patient cultures. In both patient and control myotubes, steady-state levels of respiratory chain enzyme subunits and activity of cytochrome *c* oxidase were higher than in myoblasts. In contrast to control myotubes, however, patient myotubes contained swollen and unevenly distributed mitochondria and cytochrome *c* oxidase staining was irregular. The life span of patient myotubes was dramatically shorter than of control myotubes. Our results suggest that the respiratory chain enzyme defects seen in skeletal muscle biopsies from these patients may be due to, or enhanced by, a failure of normal mitochondrial biogenesis during muscle development.

P-41
Identification of a novel mutation in the catalytic subunit 1 of the pyruvate dehydrogenase phosphatase (PDP1) gene in two brothers

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Pyruvate dehydrogenase deficiency (PDH; OMIM 312170) is a clinically heterogeneous disorder caused by mutations in the E1alpha, E3 and protein X components. PDH activity is regulated by phosphorylation: it is inactivated by PDH kinase and activated by PDH phosphatase, PDP. Patients have been reported in the literature that have a reduction in PDH phosphatase activity, but no mutation has ever been identified.

We have identified two brothers with hypotonia, feeding difficulties and delayed psychomotor development with elevated lactate levels. Both patients demonstrated deficient native PDH activity in skin fibroblasts and lymphoblasts. However, activation of the PDH complex using dichloroacetate showed almost normal PDH activity, suggesting there was a possible problem with the efficient regulation of the enzyme complex.

PDP is a dimeric enzyme that consists of a catalytic and regulatory subunit; the catalytic subunit has two isoforms which show differing dependencies to Ca^{2+} and Mg^{2+}. After sequencing the cDNA of all three genes, we identified a three-base-pair deletion in the PDP1 gene in both brothers, which was confirmed in genomic DNA. We have isolated purified human PDP1 and PDP2 proteins and showed them to be active in vitro. We are currently identifying methods by which we can restore PDH activity in the patients, both in intact cells (transfection) and in lysates (enzyme replacement).

P-42
Assigning pathogenicity to mitochondrial tRNA mutations: when "definitely maybe" is not good enough

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Mitochondrial tRNA (mt-tRNA) mutations have a wide spectrum of effect, some are tolerated others result in disease. Not all the factors that influence the pathogenicity of mt-tRNA mutations are quantified, thus predicting phenotypic effects from sequence variation in mt-tRNA genes is extremely difficult. Reliably distinguishing pathogenic mutations from neutral variations would be a major advance in the understanding of these molecules. We reviewed the literature for each mt-tRNA mutation listed as pathogenic on the MITOMAP database and we scored each according to the extent and quality of the evidence for pathogenicity.

The following conclusions could be made: (a) ~ 73% of pathogenic mutations occur in the stems of mt-tRNA;

(b) 'hot spots' for pathogenic mutations occur in both the acceptor/anti-codon stems; (c) disruption of Watson–Crick base pairing is an almost universal feature of pathogenic mutations occurring in the stem structures; (d) disruption of C–G base pairing is a significantly more common feature of pathogenic mutations than neutral variants; and (e) when pathogenic mutations occur in loop structures, they tend to occur in loops that are unusual in their size and/or influence the tertiary folding/function of the mt-tRNA.

What we have discovered will give guidance for current sequencing studies. In the long term we hope to be able to build a diagnostic tool using an artificial neural network to be available on the web that will be able to integrate information from clinical observations, biochemistry, histochemistiy and evolutionary/structural information to produce a probability that a presented change is involved in pathogenicity.

P-43

Functional alteration of cytochrome *c* oxidase by SURF1 mutations in Leigh syndrome at normoxia and hypoxia

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We performed a complex functional characterization of fibroblasts harboring different SURF1 mutations from patients presenting with severe and generalized COX defect. WB analysis revealed 85% decrease of the normal-size COX complexes and significant accumulation of incomplete COX assemblies of 90–120 kDa. Spectrophotometric assay of COX activity showed a 70–90% decrease in lauryl maltoside-solubilised fibroblasts. In contrast, oxygen consumption in patient cells revealed only a 13–31% decrease of COX activity that was highly sensitive to lauryl maltoside. ADP-stimulated respiration was 50% decreased and cytofluorometry showed a significant decrease of mitochondrial membrane potential in state 4, and a 2.4-fold higher sensitivity of mitochondrial potential to uncoupler. These data indicate that the absence of the Surf1 protein leads to the formation of incomplete, detergent-sensitive COX assemblies, which in situ maintain rather high electron-transport activity, while their H^+-pumping is impaired. As the cells with SURF1 mutations showed hardly detectable changes of respiratory rates at normoxic conditions, we analyzed their respiratory response to low oxygen. The oxygen kinetics was quantified by p50 (the pO_2 at half-maximal respiration rate) in intact coupled cells and in digitonin-permeabilized uncoupled cells. In both cases, the p50 in patients was elevated, indicating two- to threefold decreased affinity of COX to oxygen. This would mean that the depressed oxygen affinity may in vivo lead to limitations of respiration because tissue pO_2 is physiologically rather depressed. Even more pronounced effect can be expected during respiratory tract infections when the oxygen supply to tissues is limited and Leigh syndrome patients significantly worsen.

P-44

The use of muscle needle biopsy specimens in mitochondrial enzyme diagnostics

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Despite the possible use of human skin fibroblasts for diagnostic studies, muscle is the tissue of choice to study oxidative phosphorylation. A drawback is that a rather a large biopsy is needed of at least 150–200-mg wet weight being rather unattractive for the patient. To investigate if mitochondrial enzyme diagnostics is possible in smaller samples obtained by needle biopsy, we have scaled down our spectrophotometric methods for the biochemical analysis of the oxidative phosphorylation in tissue homogenates. We have determined the activities of complexes I, II, III, IV and V in about 60 needle biopsies (weighing about 10–20 mg). The mean activity of all complexes, expressed on the amount of citrate synthetase, was not different from the values obtained earlier in a group of large muscle biopsy specimens. Besides the adjustment of our spectrophotometric methods, we also adjusted our polarographic method in order to study the oxygen consumption and ATP synthesis in small, fresh muscle biopsies. In an initial study we were able to detect the oxygen consumption in a fresh needle biopsy of only about 40 mg. We conclude that it is not longer necessary to obtain large surgical biopsies for reliable mitochondrial enzyme diagnostics.

P-45
Human heart mitochondrial proteome elucidated by 2D electrophoresis and MALDI-TOF analysis

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Proteins represent the primary functional output of the cell and therefore might be expected to provide the most relevant information in biomedical research. This is particularly true when interpretation of their expression takes into account their dynamics in a specific biological context. For this purpose, two-dimensional gel electrophoresis-based proteomics (2D-proteomics) has been implemented thoroughly in recent years to address different topics in biomedical research. Also recently, Blue-Native gel electrophoresis has been applied in proteomics as a separation method due to its greater ability (compared to conventional 2D gel electrophoresis) to separate hydrophobic membrane proteins. Using both techniques, and in combination with MALDI-TOF as identification method, we have recently started to analyse the mitochondrial proteome of control human heart. At present, we have been able to identify 348 different proteins, 100 of which have already been annotated as mitochondrial. As this effort continues we are confident that much more proteins are still to be identified and, therefore, more information will be presented during the meeting. Eventually we will establish a reference map for human heart mitochondrial proteins for future comparative proteomics.

P-46
Missense mutation in mitochondrial glutamate transporter in autosomal recessive neonatal myoclonic epilepsy

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Early myoclonic encephalopathies (EME) are early-onset epileptic encephalopathies with frequent seizures in the neonatal and early infancy period. These disorders are characterized by a typical EEG pattern, namely suppression-burst, in which higher-voltage bursts of slow waves mixed with multifocal spikes alternate with isoelectric suppression phase. Affected children also present severe neurological anomalies with fatal outcome. The causes of EME are various including metabolic diseases and brain malformations. However, despite recent advances in the elucidation of the molecular bases, the cause of these disorders remains largely unknown.

Here we report the identification of a novel gene involved in autosomal recessive EME. Homozygosity mapping in an inbred Israelian family with four affected children localized the disease-causing gene on chromosome 11p15.5. This interval encompasses the SLC25A22 gene (also known as GC1) encoding one of the two mitochondrial glutamate transporter. We identified a missense mutation (Pro 206 Leu) which co-segregates with the disease and alters a highly conserved amino acid. Functional analysis shows that cultured skin fibroblasts harboring the mutation were strongly defective in the oxidation of glutamate, supporting the notion of an impaired glutamate transport across the inner mitochondrial membrane as a cause of EME.

Our data suggest that a defect in glutamate mitochondrial transport may interfere with normal neuronal function, providing new insight into the understanding of the physiopathological bases of idiopathic neonatal myoclonic epilepsy.

P-47
The incidence of a minor arc mitochondrial DNA deletion in human skin cancer and its novel use as a highly sensitive marker for sunlight exposure

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The incidence of non-melanoma skin cancer (NMSC) is increasing worldwide with more than 1 million annual cases in the USA. Our group has pioneered the use of mtDNA as a biomarker of the UVR exposure in human skin, which is the major determinant of NMSC.

We show for the first time in human skin that a rarely reported mtDNA deletion in the minor arc can be used to distinguish between constant (face and hands) and intermittent (trunk and legs) sun-exposed skin. This is important as NMSC is formed on constant exposed skin. PCR investigation of 104 skin samples from different sites of sun exposure identified the deletion in 31/42 constant sun-exposed samples which contrasts markedly ($P < 0.0001$ Fisher's exact test) with the intermittent samples (i.e. 8/42). The deletion was absent in 20 sun-protected samples. Having developed a real-time Taqman PCR assay, a higher level of the deletion occurs in constant ($n = 30$) than in intermittent ($n = 18$) sun-exposed samples ($P = 0.016$ two-tailed t test). Seventy-three percent of tumour samples ($n = 30$) also harboured the

deletion, and mutational analysis using DHPLC (Mitoscreen kit, Transgenomics) identified a somatic heteroplasmic T–C mutation at np7705.

The deletion was induced in cultured human skin cells through repetitive sublethal doses of solar UVR thereby confirming that the deletion in human skin is directly related to UVR exposure.

This novel deletion is the most sensitive mtDNA marker of sun exposure in human skin and occurs in a high proportion of NMSCs. Applications include its use as a highly sensitive UVR biomarker for early detection of NMSC development.

P-48

A homoplasmic ND5 mutation associated with MELAS: mitochondria as ATP consumers?

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Homoplasmic mtDNA variants found in patients presenting with multisystem mitochondrial disease are usually dismissed as nonpathogenic polymorphisms. We report here the first homoplasmic mtDNA point mutation with variable penetrance causing a multisystem neurological disease including MELAS. A severely affected proband and her asymptomatic mother both harboured a homoplasmic ND5 gene mutation (13565C>T) resulting in a serine to phenylalanine amino acid change (S410F). Transmitochondrial cybrid studies confirmed the pathogenicity of the mutation. Complex I-linked respiration in the mutant cybrid was reduced (37% of control) and lactate generation over 48 h in the mutant cybrid was increased (24% above control).

Mitochondrial membrane potential ($\Delta\psi_m$) in the S410F mutant cybrid was 28% less than in control cybrids and was further reduced by blocking the adenine nucleotide translocator (ANT) with 10 μM bongkrekic acid, suggesting that $\Delta\psi_m$ in the S410F mutant is partially maintained by reversal of the ANT (as in ρ0, mtDNA-less, cells). Blocking the mitochondrial ATPase with 2.5 μg/ml oligomycin caused hyperpolarisation of $\Delta\psi_m$ and decreased NADH oxidation in control cells whereas $\Delta\psi_m$ and NADH oxidation remained unchanged in the S410F mutant.

Mitochondrial calcium uptake was impaired in the S410F mutant cybrid. Thus, analysis of the S410F mutation has revealed multiple mechanisms that may contribute to its pathogenicity. Impaired mitochondrial Ca^{2+} uptake may impair the ability of mitochondria to up-regulate ATP production with increased demand. Defects in respiratory chain function causing a loss of potential may result in the mutant mitochondria functioning as ATP 'consumers' rather than 'producers'.

P-49

MIDAS, a nuclear gene product involved in the biogenesis of mitochondria in response to their dysfunction

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Mitochondrial mass is altered in response to external stimuli and mitochondrial DNA (mtDNA) damage. The biogenesis of mitochondria requires the metabolism of mitochondrial phospholipids and the expression of a large number of genes. Several transctiptional activators and coactivators have been shown to be involved in mitochondrial biogenesis in response to external stimuli. However, the mechanism of mitochondrial biogenesis in response to mtDNA damage is not understood. To investigate this mechanism, we screened the factors whose expression was enhanced in a cell line lacking mtDNA and identified a factor, MIDAS (mitochondrial DNA absence-sensitive factor). The expression of MIDAS was examined in muscle fibers of patients with mitochondrial diseases, CPEO (chronic progressive external ophthalmopelia; a large deletion in mtDNA) and MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; a point mutation in mtDNA). MIDAS was more expressed only in muscle fibers in which mitochondria were dysfunctional and accumulated, suggesting that the expression of MIDAS was enhanced in response to mtDNA damage. A majority of MIDAS localized in mitochondria with a small fraction in the Golgi apparatus in HeLa cells. To examine the function of MIDAS, we constructed HeLa cells constitutively expressing MIDAS. The increase of the total mass of mitochondria in MIDAS transfectants was revealed by three independent methods: (1) three-dimensional imaging analysis of mitochondria, (2) flow cytometry using MitoTracker Green and (3) assay of cardiolipin, a mitochondria-specific lipid. These data indicated that MIDAS is involved in the regulation of mitochondrial lipids in response to mitochondrial dysfunction.

P-50

Proteomic and genomic analysis of *Chlamydomonas reinhardtii* complex I: conserved components in eukaryotes

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The rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I) is the most intricate membrane-bound enzyme of the mitochondrial respiratory chain. A comprehensive analysis of the bovine enzyme revealed that it is composed of 45 subunits. In this work, we have first attempted to characterize the subunit content of the complex I from the unicellular green alga *Chlamydomonas reinhardtii*. Analysis by Blue-native polyacrylamide gel electrophoresis coupled to tandem mass spectrometry (MS) has allowed the identification of 30 proteins in the *Chlamydomonas* enzyme. Comparison of the known complex I components from higher plants, mammals, worms and fungi with the MS data set and the translated sequences from the algal genomes revealed that the complex I in *Chlamydomonas* might be composed of 42 proteins for a total molecular mass of about 1000 kDa.

By compiling recent data, it appeared that a set of 27 proteins are widely conserved in eukaryotic complex I. We describe here five new subunit families (ESSS, PFFD, B12, B14.7, B16.6), extending to 32 the number of widely conserved complex I components. In parallel, a variable number of subunits seems to be specific to each eukaryotic kingdom (animals, fungi or plants). However, since the degree of conservation of most of these proteins within their class is rather low, it is hard to know without additional information whether some of these proteins represent less conserved homologues, structural counterparts or are real lineage-specific components that would have been acquired or lost during the evolution in specific groups.

P-51

Functional complementation, homozygosity mapping and transcriptome analysis reveal a novel cause for respiratory chain complex I deficiency

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Complex I deficiency accounts for ~ 30% of respiratory chain disorders. To date, mutations have been identified in eight nuclear genes and six mitochondrial DNA genes encoding the most conserved complex I subunits. There appear to be a substantial number of unidentified genes causing complex I deficiency, some of which are likely to be involved in complex I assembly. We are attempting to find novel causes of complex I deficiency, initially by using complementation analysis to identify multiple patients with defects in the same gene. Of eight patients with an unknown autosomal recessive cause, two cell lines failed to complement and showed a similar profile of abnormal complex I assembly intermediates. These cell lines were studied by an integrated approach using homozygosity mapping, chromosome transfers and expression analysis of 18,000 genes in patient cell lines with oligonucleotide microarrays. This implicated a complex I subunit gene not previously associated with complex I deficiency, which was grossly underexpressed in the two patient cell lines. Both patients had homozygous mutations in this gene, one causing a splicing abnormality and the other a large deletion. This integrated approach offers promise for identifying other unknown causes of respiratory chain disorders.

P-52

A homoplasmic mtDNA mutation promotes rapid decay of deacylated mt-tRNAVal

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Homoplasmic mitochondrial tRNA (mt-tRNA) mutations are recognised as the cause of a variety of organ specific and multi-system diseases. Families harbouring such mt-tRNA mutations often demonstrate considerable clinical variability. The basis of this phenotypic disparity is poorly understood, but is thought to involve factors related to both the mutation and the nuclear background. We have investigated the molecular aetiology of the homoplasmic 1624C>T mt-tRNAVal mutation in cell lines and muscle from both mildly and severely affected individuals. Steady-state levels of mt-tRNAVal are decreased in myoblast and fibroblast cell lines from individuals harbouring the 1624C>T mutation when compared with controls. However, the steady-state levels of mt-tRNAVal in cultured cells are significantly greater than those found in muscle, where a profound biochemical defect has previously been identified. We have also demonstrated reductions in the steady-state level of mt-tRNAVal in 143B and A549 transmitochondrial cybrid cells, comparable to those seen in patient myoblasts. Thus, irrespective of the nuclear background or cell type, steady-state levels of mutated mt-tRNAVal remain low, confirming the 1624C>T mutation as the primary aetiological factor. We have shown in myoblast cultures that reduction in the steady-state level of mt-tRNAVal is due to rapid degradation of the mutated mt-tRNAVal and, furthermore, that the deacylated form of mutated mt-tRNAVal is more readily degraded than the aminoacylated species. Therefore, unlike many other mt-tRNA mutations, the 1624C>T mutation exerts its pathogenic effect by decreasing the stability of the deacylated species, and thereby limits the availability of mutated mt-tRNA for aminoacylation.

P-53
Mitochondrial DNA mutations in human colonic crypt stem cells

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Firmly established as important causes of neurological disease, the true impact of mitochondrial DNA (mtDNA) mutation in human pathology remains undetermined. In addition to the well-documented, pathogenic mtDNA mutations that are associated with classical ''mitochondrial'' phenotypes, somatic mtDNA mutations accumulate to high levels in individual cells during the course of normal ageing and though a causal link has not been established, they have been widely demonstrated in a variety of human tumours. Until recently, however, there were no data to support the accumulation of mtDNA mutations in stem cell populations.

To address this question, we have performed extensive histochemical analysis and mtDNA sequencing of human colonic epithelium, a tissue in which the progeny of a limited number of stem cells can be easily identified within colonic crypts. In this actively dividing tissue, we find extensive respiratory chain deficiency, which has led us to make the following observations:

[1] there is widespread cytochrome *c* oxidase (COX) deficiency affecting whole and partial crypts
[2] we find a high incidence of mtDNA mutation, with approximately 50% of all cells studied (both COX-deficient and COX-positive) harbouring somatic mtDNA mutation
[3] there is an increase in the incidence of mtDNA mutation, and the associated biochemical phenotype, with age and
[4] these mtDNA mutations are present in colonic crypt stem cells prior to malignant transformation.

We believe our observations in human colonic crypt stem cells are relevant to other human stem cell populations.

P-54
Regulation of nuclear-encoded mitochondrial genes in myogenesis

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Mitochondrial biogenesis requires expression of around 1000 genes localized on both the mitochondrial DNA and the nuclear genome. The pathways which lead to coordinated expression of the nuclear-encoded genes and to synchronization with the mitochondrial genome are yet unclear.

To find factors involved in gene regulation, we analyzed myogenesis of skeletal muscle cells in vitro. During differentiation of myoblasts to myotubes, mitochondrial mass and activity are strongly increased. The basic regulatory mechanisms triggering mitochondrial biogenesis were studied by analysing expression of three nuclear-encoded mitochondrial genes: expression of CytC, TFAM and COXIV is up-regulated by factors of 4–6, as studied by Western, Northern and luciferase reporter assays.

Promoter studies revealed the dependence of cytC induction on a CRE site at pos. −109 of the promoter sequence. Protein–DNA interaction analysis and changes in phosphorylation pattern of CREB proteins during differentiation gave evidence that cytC gene expression is regulated by the CREB pathway, while the role of CREB for TFAM and COXIV expression needs to be analyzed in more detail.

To further investigate common regulatory features of the analyzed promoters, the function of an unknown consensus sequence present in all three promoter was also analyzed, and proteins binding to this site were identified.

P-55
Correlation of mitochondrial copy number with cytochrome *c* oxidase histochemistry

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Heteroplasmic mitochondrial DNA (mtDNA) mutations only cause a biochemical defect when the percentage level of mutated mtDNA exceeds a critical threshold level, but it is not clear what exactly causes the loss of respiratory function for different mtDNA mutations. Is there an increase in mutated mtDNA having a dominant negative effect? Is there a decrease in the number of wild-type molecules? What is the relationship of mtDNA proliferation to this process? To address these questions we have developed a quantitative real time PCR assay, which allows the accurate determination of mitochondrial copy number down to 20 molecules. Using this assay, we have measured both the total mitochondrial copy number and the number of wild-type molecules in single muscle fibres from patients with known mtDNA mutations. Here we describe the variation in copy number

that we have seen in these patients and how it correlates with cytochrome oxidase activity.

P-56
A proteomic approach to mtDNA depletion

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Depletion of mitochondrial DNA is a disorder of mtDNA maintenance, replication and/or repair. It is characterised by a reduction in copy number of DNA molecules in affected tissues, and additional mtDNA mutations may contribute to the phenotype. This is a tissue-specific condition in which many of the affected patients present with a generalised disease involving at least the brain, muscle and/or liver, and the remaining patients with single organ disease of which 2/3 involve the muscle and 1/3 the liver. The autosomal nature of depletion suggests involvement of a nuclear factor, and complementation analysis has implicated thymidine kinase in myopathic disease and deoxyguanosine kinase in hepatocerebral disease. To further our understanding of this disease, we have performed 2D-PAGE of seven depletion patients and compared this to a control fibroblast line. Computer and mass spectrometric analysis of these data has revealed common changes occurring in these patients that may aid our understanding and diagnosis of this disease.

P-57
The significance of organic acid accumulation in paragangliomas

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Abstracts:

Following the genetic delineation of the origin of a number of paragangliomas and pheochromocytomas, it has been shown that those due to mutations in the SDH A,B or C gene resulted in the total absence of succinate dehydrogenase activity in the tumors. Although the possibility that other types of respiratory chain deficiency lead to tumor formation as well, it is striking that blockade of this particular complex, and the metabolically related enzyme fumarase, specifically causes either paraganglioma and/or pheochromocytomas, or uterine and skin leiomyomatomsis and papillary renal cancer, respectively. In the course of our biochemical studies on paragangliomas and our attempts to account for the variable consequences of complex II and fumarase loss of activity (encephalopathies or tumor formation), we found significant amount of organic acids, especially succinate, in tumoral tissues. Interestingly enough, although much higher in the tumors from patients with mutant SDH, significant levels of succinate (largely superior to the K_m of the SDH for succinate) were also observed in tumors with normal succinate dehydrogenase activity. The mechanism leading to this organic acid accumulation might well be related to the Pasteur effect (known to take place in the tumor tissues) and hypoxia, but succinate accumulation might constitute a determining step in tumor formation either by modulating the ability to control superoxide formation by the respiratory chain or by interfering in the signalling cascade for cell proliferation.

P-58
Actin brain aggregation in identical twins with dystonia and deafness: dysregulation of actin dynamics as a consequence of a bioenergetic defect

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Actin is a major cytoskeletal protein in neurons, and the dynamics of its assembly is involved in many aspects of cell motility and membrane turnover, representing a major ATP-consuming process in cells. Actin is co-transported with actin-binding proteins, including ADF and cofilin essential for rapid turnover of actin filaments in vivo, regulated through a phosphorylation process. Recently cofilin has been shown to be translocated into mitochondria, crucial for the initiation of apoptosis.

We have reported extensive actin aggregation in the brains of identical twins with progressive dopa-unresponsive generalized dystonia and deafness associated with multiple developmental abnormalities. Immunohistochemical studies revealed that rod-like inclusions in neocortex and basal ganglia were strongly immunoreactive with antibodies to ADF/cofilin proteins. Two-dimensional electrophoresis of brain extracts demonstrated increased phosphorylation of cofilin. DNA microarray studies of mitochondrial gene expression using our Mitochip and real-time quantitative PCR in brain tissues showed in-

creased gene expression of several genes including actin, cofilin, genes involved in apoptosis and mitochondrial ROS scavenging enzymes in response to increase mitochondrial ROS production. Furthermore, expression of genes involved in mitochondrial ATP production was down-regulated in brain cortex. Sequencing of the entire mtDNA did not show any abnormalities linking these cases to a nuclear defect. Because actin reorganization requires ATP consumption, a defect in mitochondrial bioenergetics may perturb cellular ATP levels and alter the phosphorylation state of cofilin. Rod formation in response to mitochondrial stress is may be a common mechanism in mitochondrial pathophysiology to protect neurons and conserve ATP by slowing actin filaments dynamics.

P-59
Detection of respiratory chain complex I activity in blood lymphocytes

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The identification of a defect in oxidative phosphorylation in a muscle biopsy is one of the complementary pillars to diagnose mitochondrial encephalomyopathy (MM) in patients. All respiratory chain complexes can be confidently measured in muscle homogenates, but obtaining a muscle biopsy is invasive and puts a heavy burden on a patient. A skin biopsy is a less demanding alternative and is often used additionally for the diagnosis of MM. A drawback is that a defect in the mitochondrial DNA may disappear during culture of the fibroblasts leading to false-negative results, and that complex I activity cannot be measured reliably due to the presence of very high rotenone-insensitive NADH-quinone oxidoreductase activity in those cells. An obvious alternative is blood lymphocytes. These cells are not often used to measure complex I activity spectrophotometrically, because the issue of the limited access of substrates to the respiratory chain complex hindering its detection is thus far unresolved, despite some recent publications suggesting the contrary.

We have developed a method to improve substrate access to complex I to enable detection of its activity in blood lymphocytes of normal donors. An initial study in affected members of a Dutch family suffering from Leber's Hereditary Optic Neuropathy (3460/ND1 mutation) indicates that a biochemical phenotype can be detected in their blood lymphocytes using the new method.

P-60
Human mitochondria possess a translation-dependent deadenylation decay pathway

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Human mtDNA is transcribed from both strands, producing polycistronic RNA units. These units are rapidly processed into discrete RNA species and immediately matured to produce mt-mRNAs and mt-rRNAs that are polyadenylated, or mt-tRNA species that carry a 3′ CCA trinucleotide. The mechanisms underlying these processes are poorly characterised and the role of polyadenylation in mammalian mitochondria is unclear.

A micro-deletion (??) has been identified in a patient presenting with mtDNA disease. Loss of the two final nucleotides of mtDNA-encoded MTATP6 removes the termination codon proximal to the processing site, generating an in-frame fusion with MTCO3. Accurate processing at this site still occurs, however, there is a marked decrease in the steady state level of the bi-cistronic mRNA encoding ATPase 8 and 6 (?? RNA14). We report how an mtDNA mutation that results in the loss of a termination codon causes instability of a mitochondrial mRNA by translation-dependent deadenylation. Based on the recent identification of a nonstop mediated decay pathway in the cytosol, a model is proposed to explain this phenomenon.

P-61
Targeting a peroxidase mimetic to mitochondria

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Reactive oxygen species (ROS) are implicated in a range of progressive neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Friedreich's ataxia. Mitochondria are the major source of ROS production and these ROS are detoxified by several antioxidant enzymes in mitochondria including glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase. Ebselen, a glutathione peroxidase mimic, can ameliorate oxidative stress that is a major cause of mitochondrial dysfunction. However, its effect is limited since it is not concentrated in mitochondria. We have developed mitoperoxidase, a mitochondria targeted derivative of Ebselen that effectively catalyses the reduction of hydroperoxides using thiols such

as glutathione and thioredoxin as electron donors. Substrates detoxified by mitoperoxidase include hydrogen peroxide, *tert*-butylhydroperoxide and mitochondrial membrane-bound phospholipids peroxides.

Mitoperoxidase is selectively accumulated into isolated mitochondria as a result of the mitochondrial membrane potential. It prevented lipid peroxidation in mitochondria and mitochondrial membranes, measured by the oxidation of *cis*-parinaric acid. Mitoperoxidase protected mitochondrial function in the presence of ferrous iron and hydrogen peroxide and reduced membrane-bound hydroperoxides following lipoxidase treatment. Hydroperoxides were not reduced in mitochondria depleted of glutathione during the membrane permeability transition or in mitochondria treated with the oxidant diamide confirming that mitoperoxidase can use glutathione as its thiol reductant. Mitoperoxidase was taken up by mitochondria within RBL2H3 cells where it delayed the onset of apoptosis induced by either 2-deoxyglucose or hydrogen peroxide. In summary, mitoperoxidase is an effective mitochondrially targeted glutathione peroxidase mimetic that protects against lipid peroxidation and has therapeutic potential in diseases involving mitochondrial oxidative damage.

P-62

Novel mitochondrial tRNATrp mutation associated with encephalomyopathy: identification using polyacrylamide gel electrophoresis coupled with matrix-assisted laser desorption/ionisation mass spectrometry

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More than 70 different point mutations in human mitochondrial tRNA genes are correlated with severe disorder, including fatal cardiopathies, encephalopathies, myopathies, and others. So far, investigation of the molecular impact(s) of mutations has focused on the affected tRNA itself by seeking structural and/or functional perturbations capable of interfering with synthesis of the 13 mitochondrion-encoded subunits of respiratory chain complexes. Here we report a fast and simple method for the structural analysis of newly identified tRNA mutations. In analogy to two-dimensional analysis, the mobility shift in native polyacrylamide gel electrophoresis (PAGE) due to a nucleotide substitution of a single-stranded transfer ribonucleic acid (tRNA) fragment serves as the first dimension for tRNA mutation analysis. Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS), as the second dimension, allows precise determination of the mass of the tRNA fragments resolved by native PAGE. Off-line combination of native PAGE with MALDI-MS is demonstrated for high-resolution analysis of mitochondrial tRNAs and its mutants. Using this method, we characterised a novel mitochondrial tRNATrp (T553C) mutation with late-onset encephalomyopathiy.

P-63

Biochemical and molecular investigations in patients with mitochondrial DNA depletion and dGK gene mutations

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Mitochondrial DNA depletion syndrome, inherited as an autosomal recessive trait, is a rare quantitative disorder characterized by a variable tissue-specific reduction in mtDNA copy number. The age of onset is usually in infancy and patients die generally before 1 year of age. Mutations in the TK2 and dGK nuclear genes, encoding for two proteins involved in mitochondrial dNTP metabolism and mtDNA replication, have been described for a small subset of patients with a myopathic or hepatocerebral form, respectively.

We investigate three patients presenting with different clinical forms of mtDNA depletion, quantified by real-time quantitative PCR. Three types of mutations in homozygous or heterozygous state were identified in the dGK gene: (1) one insertion (g.31355_31358 insGATT), for one of the two patients with an isolated hepatic form, (2) two original splice mutations (g.204 + 1 G>A and g.23884 G>A) for the patient with the typical hepatocerebral form, (3) two new missense mutations (N46S and L266R) for the third patient presenting with an unexpected reversion of an isolated hepatic form. The functional consequence of these mutations was documented in mitochondrial preparations from fibroblats by optimized dGK activity measurement.

Identification of mutations in the dGK gene and assessment of dGK activity in fibroblasts open new perspectives to correlate genotypes with phenotypes and allow accurate prenatal diagnosis in families with mtDNA depletion.

P-64

The role of the immune system in tissue-specific mtDNA segregation

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In human mitochondrial encephalomyopathies due to mtDNA mutations, there are distinct tissue-specific segregation patterns associated with different mtDNA mutations. The factors that control the segregation of mtDNA sequence variants remain poorly understood. We have studied the process of mtDNA segregation in heteroplasmic mice segregating two mtDNA genotypes, NZB and BALB. Tissue-specific and age-dependent selection of different mtDNA genotypes was observed in the liver, kidney, and spleen. In mice, mitochondrially encoded peptides can be presented as minor H antigens. We are testing whether antigen presentation of different mitochondrially encoded peptides is responsible for directional segregation of mitochondrial DNA genotypes. To address this question, we have generated heteroplasmic mice missing different components of the immune system, from peptide processing to peptide recognition. We have also characterized mtDNA segregation in different cell lineages of the spleen to test whether selection for BALB mtDNA is restricted or widespread. Different cell populations were sorted by cell surface markers and genotyped for their respective heteroplasmy level. Selection for the BALB mtDNA genotype was widespread occurring in the following cell types: B-cells, T-cells, macrophages, and granulocytes. Identifying the mechanisms responsible for tissue-specific mtDNA segregation in mice will be essential to our understanding of human mitochondrial diseases.

P-65
Alternative splicing and nonsense-mediated mRNA decay in genetic disorder of complex I

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Human mitochondrial complex I (CxI) consists of at least 46 subunits whose function is mostly unknown. The 18-kDa subunit, encoded by the nuclear NDUFS4 gene, is phosphorylated in response to cAMP promoting complex I activity. In some severe forms of encephalomyopathies, mutations affecting the expression of the 18-kDa subunit prevent the assembly of a normal functional complex and produce the appearance of defective subcomplexes. Investigations on the pathogenic mechanisms of three different homozygous NDUFS4 mutations have revealed that each mutation, although in the same gene, may act by a different mechanism. The presence of a nonsense mutation, in one patient, elicited a mechanism of mRNA degradation known as Nonsense Mediated Decay (NMD), whereas in the other two patients the steady-state amount of the transcript was normal. Our results are consistent with the occurrence of a multi-step quality control system at posttranscriptional and/or at posttranslational level to avoid the presence in the cell of abnormal polypeptides resulting from mutations in the NDUFS4 gene. NMD can be also involved in regulating the expression of natural proteins and eliminating transcripts that are generated in error by alternative splicing. Translation of such transcripts might, in fact, generate aberrant and potential harmful proteins. Many alternative splicing events are very rare and occur only in specific tissue at a specific time of development and/or under certain physiological conditions. We are investigating on the occurrence and role of alternative transcripts of NDUFS4 gene and on the isoforms of the NDUFS4 protein.

P-66
Differences in assembly or stability of mitochondrial OXPHOS complexes in inherited complex I deficiency

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Mutations in NADH:ubiquinone oxidoreductase (complex I) nuclear structural genes are the most common cause of isolated complex I enzyme deficiencies. The cell biological consequences of such mutations remain poorly understood. We have used blue native electrophoresis to study how different nuclear mutations affect the integrity of mitochondrial OXPHOS complexes in fibroblasts from complex I-deficient patients. Our results show an important decrease in the levels of intact complex I in patients harboring mutations in nuclear-encoded complex I subunits, indicating that complex I assembly and/or stability is compromised. Different patterns of low molecular weight subcomplexes are present in these patients, suggesting that the formation of the peripheral arm is affected. Mutations in complex I genes can also affect the stability of other mitochondrial complexes, with a specific decrease of fully assembled complex III in patients with mutations in NDUFS2 and NDUFS4. These results suggest a physical interaction between both complexes that could be mediated through these subunits. We have extended the analysis to patients with an isolated complex I deficiency in which no mutations in structural subunits have been found. In this group, we can discriminate between complex I assembly and catalytic defects attending to the fact whether there is a correlation between assembly/activity levels or not. This will help us to point more selectively to candidate genes for pathogenic mutations that could lead to an isolated complex I defect.

P-67

Evaluation of assays for OXPHOS complexes in mitochondria from fibroblasts and muscle homogenates: pitfalls and limitations

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Biochemical approaches are necessary for the diagnosis of defects of the oxidative phosphorylation system (OXPHOS). Neither general diagnostic guidelines nor uniform methods are available; therefore, each lab has to establish their own methods and reference values.

We evaluated and established methods to assay OXPHOS activities. Maximal activity for CI-III required sonication of isolated mitochondria, in contrast to CIV that is susceptible to inactivation by mechanical disruption. In muscle homogenates, sonication led to progressive loss of enzymatic activities. Further disruption techniques were necessary to obtain optimal results: CI, hypotonic shock in H_2O; CII, three cycles of freezing in solid N2; CIII and CIV, *N*-dodecylmaltoside present in the reaction buffer.

The activities of muscle homogenates as well as of mitochondria from fibroblasts were stable during several months. The intra-/interassay variabilities for muscle homogenates (%) are: CI, 2.4/10; CII, 6/12; CIII, 19.5/23; CIV, 16/20.

The higher values of CIII and CIV are rather due to methological problems than to activity loss: the inhibitory effect of antimycin A varies due to different cytochrom C batches. CIV activity is very sensitive against homogenisation. Another problem is the fast initial burst of activity of CIV, followed by a much slower reaction rate. Enzyme assays were linear with respect to protein over a range of 5–12 μg mitochondrial protein for CI and CII, 5–20 μg for CIII and 1.5–12 μg for CIV. For muscle homogenates 10–90 μg protein for CI, CII, CIII and 5–12 μg for CIV have to be used to be in the linear range.

P-68

Detection and measurement of the mitochondrial A3243G and A8344G mutations in samples from patients and maternal relatives: observed intertissue repartition profiles and potential use of quantitative values, ratios or indexes

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Aim, material and methods: The proportion of mutated mitochondrial DNA species in different noninvasive biological samples (blood, urinary, buccal or hair cells) from patients with heteroplasmic mitochondrial DNA mutations or their maternal relatives is an easily available parameter. Forty-three independent occurrences of the A3243G and five independent occurrences of the A8344G have been diagnosed in our laboratory. We collected multiple samples from as many mutation carriers as possible. Two quantification methods were assessed by using a heteroplasmic standardized reference material for the A3243G mutation. We measured mutation loads in all samples, calculated intertissue ratios, and searched to what extent observed quantitative differences among interindividual result patterns may be relevant to explain qualitative differences in the natural history of disease among carriers, and may be of practical use in medical practice.

Results: Intertissular distribution patterns are quite different for the A3243G and the A8344G mutations, although there are individual exceptions. The average proportion of the A3243G mutation in urinary cells is twice that in blood, which is relevant for diagnosis practice. There is a clear correlation of global mutation load and the presence of symptoms but a predictive index is not easily defined. The threshold for symptoms seems lower and less defined for the A3243G mutation than for the A8344G.

Conclusion: The measurement of mutation load of A3243G or A8344G is easy to perform in either biopsy-obtained or noninvasive samples. Further analyses including new cases will help making it more useful in clinical practice.

P-69

Elevated production of reactive oxygen species in fibroblasts from patients with deficiencies in ATP synthase

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Genetic defects in enzymes of oxidative phosphorylation (OXPHOS) cause a broad spectrum of mitochondrial encephalomyopaties. Apart from diminished ATP production per se, elevated levels of ROS generated by defective OXPHOS complexes are implicated in pathogenic mechanism of mitochondrial diseases. This view is supported by measurements of ROS production by mitochondria of patients with complex I defects and also by the data from mice with knock-out in MnSOD gene. In our work we used fibroblasts from patients with deficiencies in ATP synthase to study consequences of OXPHOS mutations on

an in vivo ROS production, levels of cellular glutathione and mitochondrial membrane potential. We established methods for measurement of ROS production (with carboxy-H2DCFDA) and changes in mitochondrial membrane potential (with TMRM) on confocal microscope (Leica TCS SP 2) and flow cytometer (BD FASCScan) with the possibility to relate both signals to mitochondrial mass, determined by mitochondria selective probes MitoTracker DeepRed or MitoTracker Green. We also used fluorescence plate-reader (Wallac Victor2) for determination of ROS production on intact or digitonin-permeabilized cells and for determination of glutathione levels (with monochlorobimane). We found that ATPase-deficient cells produce significantly higher amounts of ROS than controls while levels of main cellular ROS scavenger glutathione are only mildly decreased. High mitochondrial membrane potential caused by defective discharge of proton gradient by ATP synthase seems to be important for increased ROS production in patient cells as uncoupler (FCCP) reduced both mitochondrial membrane potential and ROS production.

P-70
Evolving phenotype in pyruvate dehydrogenase complex deficiency

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Pyruvate dehydrogenase complex (PDHc) is an intramitochondrial multienzyme complex essential for the aerobic oxidation of glucose. The disorder is genetically heterogeneous, however, the majority of patients with a PDHc defect have abnormalities in the major catalytic and regulatory subunit, E1 alpha, which is encoded on the X chromosome. The clinical spectrum of PDHc deficiency is heterogeneous, particularly in heterozygous females, and therefore the diagnosis may be difficult. The phenotypic presentation generally includes lactic acidemia, muscle hypotonia, central nervous system involvement, developmental delay, seizures and characteristic dysmorphic features. Patients may have other congenital malformations including corpus callosum hypoplasia or ventricular septal defect. We report on four patients with a PDHc defect, one of whom carries a previously undescribed mutation in the gene coding for the E1 alpha subunit of the enzyme complex. The patients, two girls and two boys, demonstrated the following unique clinical symptoms consecutively: congenital epilepsy, congenital cataract, colobomas with vermis aplasia, and one with a complex cyanotic heart malformation.

P-71
In vivo mitochondrial complementation preventing individuals from respiration deficiency by pathogenic mutant mtDNA

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Mammalian mitochondria are dynamic organelles, repeating fusion and fission. It has been difficult, however, to show the occurrence of extensive complementation between the fused mitochondria in mammalian species, because sequence of mtDNA throughout an individual tends to be uniform owing to its strictly maternal inheritance. We previously showed the existence of extensive in vivo mitochondrial complementation in Mito-Mice, mice carrying pathogenic mutant mtDNA with large deletion. We observed the uniform distribution of either COX-positive or COX-negative mitochondria in any single cells of the Mito-Mice. However, this evidence was not unambiguous, since our experiments did not distinguish endogenous and exogenous wild-type mtDNAs. This study resolves this point with newly created Mito-Mice carrying endogenous wild-type mtDNA of *Mus spretus* and exogenous pathogenic deleted mtDNA from different mouse subspecies, *M. musculus domesticus*. The results provide unambiguous evidence for in vivo complementation between endogenous and exogenous mitochondria. The mitochondrial complementation could correspond to a very unique and effective defense system of the highly oxidative organelles for preventing individuals from expressing mitochondrial dysfunction caused by mtDNA lesions, which have been continuously created by oxidative stresses.

P-72
Leber's Hereditary Optic Neuropathy mutations alter DNA replication and Complex I transcripts

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We carried out an oligonucleotide microarray experiments in neural NT2 cybrids to better understand the neural-specific consequences of the LHON mutations, which we had previously observed to produce increased mitochondrial ROS and a defect in cellular proliferation. Several things were clear from the microarray analysis. First, LHON mutations caused inhibition of many more genes than were activated. Second, the biochemical path-

ways affected were extremely cell-type-dependent. Two main mechanisms were suggested by the analysis of NT2 cybrids. First, there was only overexpression of one nuclear-encoded subunit of complex I observed, the NDUFS4 transcript, which holds an iron–sulfur cluster (ISC). The observation is consistent with the hypothesis that the LHON mutations cause a structural change which increases ROS production and damages the ISC bound by NDUFS4 specifically; this hypothesis is consistent with our previous data that demonstrated a LHON-specific increase on mitochondrial superoxide and peroxide production which was inhibitable by rotenone. We also observed the LHON-dependent inhibition of multiple genes involved in DNA replication and cell cycling. These results were consistent with our earlier observation that LHON neural cybrids were deficient in cellular proliferation and differentiation. Thus, microarray analysis suggests two biochemical bases for LHON-dependent pathobiochemistry and pathophysiology.

P-73
A novel heteroplasmic G14739A mutation in the mitochondrial tRNAGlu in a myopathic patient

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Case: At the age of 7 years the girl was introduced to our gastroenterologist due to inappetance and weakness. Slightly increased GOT, LDH and CK levels and a discrete muscular atrophy were detected. Bicycle-ergometry showed pathological increase of lactate (from 2.4 up to 8.0 mmol/l) already after short exercise load which exhausted the patient. In EMG a clear myopathic pattern was seen. Therefore, muscle biopsy was performed.

Results: Functional biochemical investigation of native mitochondria investigated by respirometry and substrate oxidation showed normal values relative to the amount of tissue and protein, respectively; relative to citrate synthase, all values were approximately 40% reduced. A shifted pattern was found in oxidative phosphorylation enzymes with high citrate synthase and complex II. Histochemically numerous ragged-red and cytochrome *c* oxidase negative fibres were found. Electron microscopy revealed irregular mega-mitochondria with tubular structures and focally subsarcolemmal glycogen accumulations. Genetic screening of the tRNAs and rRNAs of the mitochondrial genome with denaturing HPLC showed an abnormal result in the region of the tRNAGlu where a heteroplasmic exchange of G14739A was found in sequence analysis. The mutation was present at circa 90% in muscle.

Discussion: This girl presents with a relatively mild defect in the mitochondrial energy metabolism and unspecific symptoms. Ergometry, EMG, morphology, biochemistry and finally the presence of a heteroplasmic mutation in a mitochondrial tRNA clearly defined the presence of a mitochondrial myopathy. In the tRNAGlu, previously, only one other mutation (T14709C) was reported, which was associated either with mitochondrial myopathy, deafness or diabetes.

P-74
Investigating mitochondrial DNA heteroplasmy in the female germline

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Mitochondrial DNA heteroplasmy frequencies are known to exhibit wide variation during the matrilineal inheritance of mitochondria. It has been shown that the enormous intergenerational variance of mtDNA heteroplasmy can be accounted for by a rapid and random expansion from a small, restricted pool of mtDNA in the female germline. Although endpoint observations of considerable heteroplasmy variances have been documented, the factors that are involved in this process have not yet been investigated.

We hypothesize that mitochondrial DNA copy number and respiratory chain function are important factors during this critical genetic bottleneck. Using the Cre-lox recombination system in our previously established heteroplasmic mouse model, we test this hypothesis by knocking out floxed m-TFAM in a lineage-specific manner using transgenic mice that express Cre-recombinase from a TNAP or ZP3 promoters. Tissue nonspecific alkaline phosphatase (TNAP) expression is confined to the primordial germ cells (PGC) and Zona Pellucida 3 (ZP3) is expressed in the growing oocyte. The excision efficiency of m-TFAM by the Cre-recombinase is monitored using a double reporter transgene, Z/AP. Using an Oct4-GFP transgene, whose expression is largely restricted to PGCs, FACS sorting of PGCs allows for the analysis of heteroplasmy frequency in the female germline throughout its development.

P-75
The effect of "atypical" antipsychotics on the OXPHOS system in peripheral blood mononuclear cells

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Background: A limitation in the use of classical neuroleptic drugs is the eventual appearance of extrapyramidal symptoms. Many experimental studies in rats have related these symptoms with a defect in OXPHOS, specially with Complex I. One of the advantages of the more recently introduced ''atypical'' neuroleptics is a lesser incidence of movement disorders.

Objective: To study the OXPHOS system in peripheral blood mononuclear cells (PBMCs) of psychotic patients without signs or symptoms of extrapyramidal dysfunction either in nontreated (naïve) or treated with risperidone or clozapine, two frequently used atypical neuroleptic agents.

Materials and methods: PBMCs from naïve schizophrenic patients ($n = 15$) as well as from patients under chronic treatment with risperidone ($n = 17$) or clozapine ($n = 16$) were collected. Eight naïve patients were reanalysed 6 months after initiating treatment with risperidone. Respiratory activity was polarographically determined in intact cells and after membrane permeabilization followed by the addition of pyruvate-malate, glutamate-malate or succinate as substrates. Absolute enzyme activities (complexes I through V of the OXPHOS system) were spectrophotometrically quantified.

Results: Forty-eight patients were analysed. Specific activity of Complex I (80–90% rotenone-NADH:ubiquinone oxidoreductase sensible activity) was reliably measured. We did not find differences between naïve, risperidone or clozapine-treated patients. However, when naïve patients were reanalysed after 6 months of treatment, a decrease in oxidation of pyruvate-malate and glutamate-malate could be demonstrated.

Conclusions: The importance of a decrease in complex I substrate oxidation in a nontarget tissue for neuroleptics remains to be established.

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P-76

Point-mutations in tRNASer(UCN) lead to conformational changes: evidence for altered tertiary structure and additional posttranslational modification

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MtDNA point mutations are underlying a tremendous number of different clinical phenotypes. Beyond segregation, heteroplasmy and threshold, a variety of different pathomechanisms are caused by these mutations and it is tempting to speculate that these also contribute to the clinical variability. However, still not enough is known why the change of a single base pair is ultimately either classifiable as a simple polymorphism or is leading to often serious disease. We have studied the consequences of two pathogenic point mutations (T7512C and G7497A) in the tRNA Ser (UCN) gene using osteosarcoma cell cybrids incorporating both mutations at almost homoplasmic levels, and have obtained evidence for conformational changes caused by either mutation. If run under non-denaturating conditions, we detected on Northern blots an altered electrophoretic mobility of both tRNAs. Furthermore, we introduced both mutations into synthetic tRNA molecules produced by in vitro transcription. With the in vitro transcribed molecules we found that the G7497A mutation alone was sufficient to cause an altered electrophoretic mobility, while T7512C was not. However, in both cases the mobility of the synthetic molecules was still different compared to the endogenous molecules. We conclude that the G7497A mutation per se is leading to a conformational change, while within the cell either mutation causes additional posttranslational modifications.

P-77

Normal mitochondrial creatine kinase in human creatine depletion (GA)

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Mitochondrial creatine kinase (mtCK) is located in the mitochondrial intermembrane space and phosphorylates creatine (Cr) to phosphocreatine (PCr). Recent reports have addressed the role of mtCK in the functional coupling of mitochondrial oxidation and phosphorylation.

In myopathies featuring modestly decreased Cr content, sarcomeric mtCK is reportedly decreased (muscle dystrophy) or increased (mitochondrial myopathies). Furthermore, an accumulation of intramitochondrial mtCK inclusions is seen in some mitochondrial myopathies. However, the regulation of mtCK with regard to cellular Cr content is not well understood.

Gyrate atrophy (GA) is a rare inborn error of metabolism featuring Cr depletion in muscle and brain due to inhibition of Cr synthesis in the kidney. Thus, GA constitutes a model for Cr human depletion.

We investigated mtCK adaptation to human muscle creatine depletion.

Muscle biopsy samples of four male GA patients (age 19–49 years) with biochemically verified 50% depletion of Cr, PCr and ATP were analysed by Western blot with antibodies against human sarcomeric mtCK. Samples from

healthy age- and physical activity-matched men served as controls.

Despite a marked decrease in Cr and PCr concentrations, sarcomeric mtCK content was similar in GA patients and controls.

Thus, in conclusion, our data indicate that modest decreases in cellular Cr, PCr and ATP concentrations per se do not affect mtCK protein content in human skeletal muscle.

P-78
Carnitine palmitoyltransferase II deficiency: biochemical and mutation analysis in patients detected by tandem mass spectrometric acylcarnitine profiling

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Mitochondrial carnitine palmitoyltransferase II (CPT II) deficiency is the most common inherited disorder of lipid metabolism in adults. Patients suffer from recurrent myoglobinuria, muscular weakness and myalgia, triggered by exertional exercise, cold, infection, and/or prolonged fasting. We have previously shown that tandem mass spectrometric serum acylcarnitine profiling is a sensitive, rapid and noninvasive tool for detection of CPT II deficiency (Gempel et al., 2002). Seven newly diagnosed patients were further characterized by measurement of CPT II activity in leukocytes and/or muscle biopsy and by mutation analysis of the CPT2 gene. The most sensitive (C16 + C18:1/C2) acylcarnitine ratio was clearly elevated in all patients (range: 0.119–0.263; reference range: 0.011–0.048). CPT II activity in leukocytes as measured in the backward direction and inhibition of CPT I by 5% Triton X-100 ranged from 0.01 to 0.46 nmol/min/mg protein (reference range: 1.0–2.5). A combined PCR-RFLP and gene sequencing approach revealed six different pathogenic mutations which have been previously described in association with the muscular phenotype of CPT II deficiency. Two patients were homozygous for the common S113L mutation, while three patients had an S113L mutation in combination with E487K (two brothers) or 1238_1239delAG/F448L. The second most prevalent mutation P50H was found in one patient together with the R124X mutation. The seventh patient was homozygous for the R161W mutation. In conclusion, we show that CPT II deficiency can be safely and rapidly diagnosed from a small blood sample by a combined tandem mass spectrometric, biochemical and genetic approach.

P-79
Utility of real time qPCR in molecular diagnosis of mitochondrial DNA disorders

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Molecular diagnosis of mitochondrial DNA (mtDNA) disorder is usually focused on point mutations and large deletions. PCR/RFLP or PCR/ASO (allele specific oligonucleotide) dot blot analysis for point mutations and Southern blot analysis for large deletions are the common methods used. These procedures require multiple steps including PCR, restriction enzyme digestion, and hybridization. To measure mutant heteroplasmy, radioactive labeling and quantitative analysis of the signal intensities are necessary. We developed real time quantitative PCR (RT qPCR) method to detect and quantify the mutation in one step. For point mutations, allele-specific primers for either the wild-type or the mutant target sequences are designed (ARMS RT qPCR) for PCR, and the copy number of target sequence was measured. For deletions, primers that amplify the region that is always not deleted and the region that is present in >95% of the deletion molecules so far identified are used. Inclusion of a nuclear reference gene allows the measurement of copy number ratio of mtDNA to nuclear DNA. Using this method, a point mutation present at as low as 0.1% was detected in a single-step reaction. In addition, multiple deletions with mtDNA depletion can be simultaneously detected and quantified by RT qPCR. Furthermore, we were able to identify a patient who had about 92% deletion mutant mtDNA in muscle but her mtDNA was amplified ninefold that resulted in mild Kearns–Sayre syndrome. Our data demonstrate that RT qPCR provides an effective one-step, qualitative and quantitative analysis for molecular diagnosis of mitochondrial DNA disorders.

P-80
Complex I assembly in human cells

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The 46-subunit-containing complex I (CI) is the largest complex of the oxidative phosphorylation (OXPHOS) system. The assembly of this complex is still poorly understood and insight in the assembly will provide information about the nature of the CI deficiencies and

might reveal the possible disease mechanisms. Therefore, we studied the assembly of complex I in human cells. We used cells devoid of mitochondrial DNA to test whether subcomplexes could be formed. In such a system, no fully assembled complex I can be formed because the essential ND subunits are lacking. We found, however, that still subcomplexes could be formed. To further address the nature of the subcomplexes, we blocked complex I assembly by inhibiting mitochondrial protein translation with the drug doxycycline. After releasing this block, CI resumes and assembly intermediates can be observed. By analysis of the distinct subcomplexes we propose that assembly of complex I occurs in distinct steps and is a semi-sequential process (subcomplexes are formed which are joined together to form a fully assembled complex). The membrane part of the complex is separately formed in distinct steps of which the 39-kDa subunit forms a subcomplex to which ND1, ND6 and the 15-kDa subunit are subsequently added. To this subcomplex another subcomplex containing the B17 subunit is assembled. A hydrophilic subcomplex is formed by the 30-kDa subunit to which subsequently several subunits are added. At a late stage this subcomplex is joined to the membrane arm and the holo-complex I is formed.

P-81
Reduced efficiency of 3′ end cleavage and CCA addition with mutant human mitochondrial tRNAs could contribute to pathogenesis

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Over 150 mutations in the mitochondrial genome are associated with human mitochondrial disease. Two thirds of them fall in tRNA genes, which constitute only one-tenth of the mitochondrial genome. Twenty-two tRNAs, one for each of 18 amino acids and two for Ser and Leu, punctuate the 13 mRNAs and 2 rRNAs in long polycistronic transcripts with practically no spacers, requiring precise endonucleolytic excision at both ends. Furthermore, CCA is not transcriptionally encoded, and must thus be added post 3′ end cleavage. Using unmodified transcripts, we have investigated reaction efficiency and kinetics of mitochondrial pre-tRNA 3′ end cleavage and CCA addition to test whether deficient tRNA 3′ end maturation correlates with mitochondrial pathology. The A7445G substitution in tRNASer[UCN], associated with non-syndromic deafness and suggestively located one nucleotide on the 3′ side of the 3′ end cleavage site, cannot be processed by 3′-tRNase. Of the nine tested pathogenesis-associated tRNAIle mutations, four reduce 3′-tRNase cleavage efficiency ~10-fold, all due to reduced V_{max}. One of them (A4309G) causes a pronounced local change in secondary structure. Three pathogenesis-associated mutations in tRNALeu[UUR] reduce 3′-tRNase cleavage efficiency, also through reduced V_{max}. Strongest ($\sim$ 3-fold) reductions were observed with mutations A3302G and C3303T on the 3′ side of the acceptor stem. The C3303T substitution reduced efficiency of CCA addition >5-fold, but due to increased K_M rather than reduced V_{max}. If the intramitochondrial concentration of tRNA processing enzymes is limiting, the observed reductions in reaction efficiencies, although modest, could contribute cumulatively to mitochondrial pathology.

P-82
Mitochondria and the eye: ageing and disease

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One of the most common organs affected in mitochondrial disease is the eye. The clinical features include ptosis, external ophthalmoplegia, optic atrophy, pigmentary retinopathy and cataract. These symptoms are often severely disabling with loss of vision being a major concern.

The reasons for the involvement of particular ocular tissues in mitochondrial disease are not known and until now morphological studies have been hampered by a limited supply of tissue to investigate. In particular, fresh frozen tissue for the investigation of the histochemical and molecular genetic defects in different ocular regions has been limited.

We have had the opportunity to collect ocular tissue and in some cases whole eyes from patients and control subjects (donor eyes for corneal transplants). We have shown:

1. In patients with CPEO, the incidence of COX-deficient extraocular muscle fibres is greater than that in skeletal muscle.
2. Normal ageing results in an exponential rise in the incidence of COX-deficient photoreceptors and ciliary epithelial cells.
3. In two patients with mtDNA disease (3243 A>G, 14709 T>C mutations), we have looked at all eye tissues. The most marked changes in the patient with 3243 A>G mutation was loss of ganglion cells, compatible with optic atrophy, whereas in the patient with 14709 T>C mutation the ocular changes involved the ciliary epithelium and retinal pigmented epithelium.

The tissues of the eye are heavily dependant on oxidative phosphorylation for their energy supply and the

affected tissues reflect this both in ageing and in mtDNA disease.

P-83
Complex I-caused dysfunction in mitochondria of human tumors and rat rhabdomyosarcoma

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Mitochondria seem to play an important role in energy metabolism of tumor cells. The aim of this work was to characterize mitochondrial function in human tumors [squamous cell carcinoma (SCC), parotis Warthin's tumor (PWT)] as well as in an experimental rat rhabdomyosarcoma (RS) in comparison to tonsil mucous membranes (MM) or muscle(M).

For that purpose we adapted the skinned fiber technique, previously used for investigation of muscle mitochondria only, to tumors and mucous membranes. Scaling the pyruvate-dependent respiration on the succinate respiration measured in the same sample (SRPR), we obtained a parameter that is independent from the amount of mitochondria. SRPR was found to be $120 \pm 18\%$ in normal human muscle and $132 \pm 12\%$ in healthy tonsil mucous membranes but was reduced in tumors (SCC, $64 \pm 15\%$; PWT, $21 \pm 13\%$; RS, $54 \pm 23\%$). These decreased CI-dependent respiratory rates were at least in part caused by decreased activities of CI and CI + III. Moreover, in RS the rotenone inhibition of pyruvate-dependent respiration was significantly reduced as it was deduced from the higher level of rotenone-insensitive respiration scaled to succinate-dependent respiration (RS, $35 \pm 11\%$; rat skeletal muscle, $20 \pm 13\%$). This was paralleled by an increased level of rotenone insensitive NADH oxidation in relation to the total activity (RS, $15 \pm 8\%$; rat muscle, $80 \pm 7\%$). In addition, the leak respiration was clearly higher in RS compared to rat muscle.

Data demonstrate a complex I-caused functional impairment of mitochondria in three different types of tumors (SCC, PWT, RS). These special metabolic properties of tumor mitochondria could be used for selective therapeutic concepts.

P-84
Significant increase in mtDNA copies per cell in PBMC from patients after 2 months on simvastatin therapy

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Frequently reported adverse effects of statins, compounds for the treatment of hypercholesterolemia, are muscle pain/weakening ($\sim$ 10%) with usually elevated creatine kinase levels and in some cases liver failure or rhabdomyolysis. Studies have shown that statin treatment also affects the morphology of mitochondria and results in the presence of red-ragged fibers. We investigated whether statin treatment resulted in changes in mtDNA in PBMC, using a real-time NASBA assay quantifying mtDNA and nuclear DNA in a one-tube format. A group of seven individuals with elevated cholesterol levels were treated for 2 months with 40-mg simvastatin daily, whereas a group of six individuals with elevated triglyceride levels were treated with 100-mg ciprofibrate daily. A sample was taken at day 0 and after 2 months and analyzed for mtDNA content in a blinded way. There was no significant change observed in mtDNA over time in the control group on ciprofibrate ($P = 0.292$), while the group on simvastatin showed a significant increase from 361 to 429 mean copies mtDNA per cell ($P = 0.032$). Statins inhibit HMG-CoA reductase, eventually resulting in the reduction of cholesterol, as well as a reduction of intermediates in the cholesterol biosynthesis pathway like isopentyl pyrophosphate, which also acts as precursor for coenzyme Q10 (Co-Q10). Co-Q10 plays an important role in the electron transport of the oxidative phosphorylation in mitochondria and depletion might lead to disorders in the energy metabolism. We hypothesize that depletion of Co-Q10 results in increased levels of mtDNA possibly to facilitate an increase of mRNA encoding for proteins, such as the COX family of proteins, involved in the oxidative phosphorylation.

P-85
Altered nucleoside metabolism in MNGIE generates atypical multiple deletions of mitochondrial DNA

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder. In MNGIE,

skeletal muscle typically shows histochemical and biochemical abnormalities of mitochondrial respiratory chain enzymes while tissues and cultured cells have revealed depletion, multiple deletions, and site-specific point mutations of mitochondrial DNA (mtDNA). MNGIE is caused by mutations in the gene encoding thymidine phosphorylase (TPase) and, as a consequence, TPase activity is drastically reduced in patients. TPase is a cytosolic enzyme required to maintain nucleoside homeostasis. Due to the loss of TPase function, MNGIE patients have markedly elevated plasma levels of thymidine and deoxyuridine. We have hypothesized increased levels of intracellular thymidine and deoxyuridine cause imbalances of mitochondrial nucleotide pools that, in turn, lead to the mtDNA abnormalities. DNA from autopsy tissues from three MNGIE patients and biopsied skeletal muscles from six were studied. We detected significant depletion of mtDNA in liver. We identified five major forms of mtDNA deletions (including the ''common'' deletion) in skeletal muscle of MNGIE patients. The nicotinamide adenine dinucleotide dehydrogenase (ND) 5 gene is a hot-spot for these rearrangements. Direct repeats (DRs) and imperfectly homologous sequences appear to mediate the formation of mtDNA deletions. A novel aspect of the mtDNA deletions in MNGIE is the presence of microdeletions at the imperfectly homologous breakpoints. The positions of the breakpoints within the imperfectly homologous sequences suggest that branch migration occurs and imply that intramolecular homologous recombination in nonreplicating molecules is more likely than slipped mispairing or illegitimate elongation during replication.

P-86

Lack of gastrointestinal symptoms in a 60-year-old patient with MNGIE

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MNGIE is an autosomal recessive disorder due to mutations in the thymidine phosphorylase (TP) gene characterized by gastrointestinal dysmotility, ptosis, PEO, peripheral neuropathy, and leukoencephalopathy.

Case report: A 60-year-old female had bilateral ptosis since childhood and since the age of 2 years she had tremor. She was 145-cm high and 41 kg in weight at age 60. Neurological examination showed bilateral ptosis with severe bilateral external ophthalmoplegia, and generalized areflexia tremor. Cardiologic examination revealed a mild thickening in the septum heart muscle. She never complained from gastrointestinal symptoms. A radionuclide gastric emptying functional test showed mild gastroparesis. Venous lactate was elevated. Brain MRI revealed diffuse hyperintense signal affecting cerebral hemispheres, midbrain and pons.

Results: Muscle biopsy showed RRF-COX-negative result. Muscle respiratory chain complexes were decreased. Southern blot analysis failed to reveal macroscopic alterations of mtDNA. Biochemical analysis showed negligible TP activity in buffy coat and high plasma levels of thymidine and deoxyuridine. We identified four heterozygous nucleotide changes in the TP gene: c.228G>A (M76I), c.847C>G (H283D), c.1311G>A (W437X) and the A465T polymorphism. Molecular analysis in the proband's daughter and sister demonstrated that the patient is a compound heterozygote.

Conclusions: All reported MNGIE patients reported so far showed gastrointestinal symptoms at some point in their lives. The patient reported here has only subclinical GI involvement at age 60, even though the onset of her symptoms was in the childhood. Therefore, biochemical diagnosis should always be performed to unequivocally exclude this disorder in patients with features suggestive of MNGIE, even in those lacking gastrointestinal symptoms.

P-87

Sequential dHPLC analysis of a clonally expanded mitochondrial tRNALeu(UUR) mutation (G3242A) in a patient with myelodysplastic syndrome (preleukemia) during successful treatment with thalidomide

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In bone marrow cells of patients with myelodysplastic syndrome (MDS), mitochondria often show ultrastructural abnormalities, including pathological iron accumulation in the mitochondria of erythroblasts. This suggests that mitochondrial dysfunction contributes to the pathophysiology of MDS. We are finding clonally expanded somatic mutations of mtDNA in the hematopoietic system of patients with MDS, using heteroduplex analysis with denaturing HPLC (Transgenomic WAVE™ system).

In a patient with refractory anemia with excess blasts (RAEB), heteroduplex analysis indicated that 40–50% of mtDNA molecules in the bone marrow (BM) carried a novel G3242A mutation in the mitochondrial transfer RNA Leu(UUR). Surprisingly, the mutation was not detectable by heteroduplex analysis in the peripheral blood (PB). However, the rare circulating CD34+ progenitor cells, selected by immunomagnetic beads, harbored the mutation with a proportion of approximately 50%. In hematopoietic colony assays, CD34+ cells from BM and PB yielded only colonies with wild-type mtDNA. These results indicate that the G3242A mito-

chondrial tRNA mutation in CD34+ cells was associated with a maturation defect that contributed to ineffective hematopoiesis.

The patient was treated with thalidomide which, through ill-defined mechanism of action, achieved marked hematological improvement, with cessation of transfusion requirement. In parallel, the proportion of mutant mtDNA in the bone marrow decreased from 50% in October, 2002, to about 5% in November, 2003. While the diminished heteroduplex peak on dHPLC analysis correlated with hematological improvement, heteroduplex analysis, with an in vitro detection limit of 3% mutant mtDNA in the present case, is not sensitive enough for minimal residual disease detection.

P-88
The functional analysis of an atp12 mutation in yeast

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Complex V (ATP synthase) of the respiratory chain couples the proton gradient, generated by the respiratory chain, to ATP synthesis. The ATP12 gene product is one of the proteins required for assembly of part (F1 component) of the ATP synthase.

Here, we report on the functional effect of a W94R mutation, present in the ATP12 assembly gene of a patient with an ATP synthase decreased activity. Multicopy plasmid constructs containing the human and yeast wild-type ATP12 gene, as well as the human mutation W94R and the yeast counterpart W103R, were prepared. These constructs were introduced in a *Saccharomyces cerevisiae* W303 host strain deprived of this ATP12 function (W303DATP12), and as such respiratory-deficient. ATP synthase activity of purified individual yeast transformants was assayed by growth studies on a non-fermentable carbon source (ethanol-glycerol) and by Blue Native Polyacrylamide Gel Electrophoresis stained for ATP synthase catalytic activity. WT plasmid constructs rescue the respiratory defect of a yatp12 mutant strain.

Growth on a non-fermentable carbon source such as ethanol-glycerol was strongly impaired for the human W94R mutant as compared to controls. However, the growth of the yeast W103R mutant strain was normal on ethanol-glycerol. Blue Native Polyacrylamide Gel Electrophoresis studies are in progress.

Our yeast complementation studies showed clearly that the human W94R mutant does not confer the respiratory competence to a W303DATP12 yeast strain and is most probably the cause of the complex V dysfunction in our patient.

P-89
The roles of SCO1 and SCO2 in mitochondrial copper delivery to cytochrome *c* oxidase

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Although it is clear that both SCO1 and SCO2 play a critical role in cytochrome *c* oxidase (COX) assembly in humans, their molecular functions remain unknown. Molecular genetic analyses of SCO1 and SCO2 patient cell lines and tissues were therefore undertaken in order to further characterize their relative roles in mitochondrial copper delivery to COX. Mutations in either SCO1 or SCO2 had modest effects on the protein levels of other factors known to participate in the mitochondrial copper delivery pathway. With the exception of COX17, none of these factors, when overexpressed, were able to rescue the observed COX deficiency in either patient background. Parallel experiments involving SCO1 and SCO2 suggest that each fulfills a unique yet inter-dependent function in the delivery of copper to COX during holoenzyme assembly. Complimentary analyses in SCO2 patient tissues further suggest a potential mechanism that explains the relative roles of SCO1 and SCO2 in the biogenesis of the CuA site contained within COX II. This model may also provide some insight into the tissue-specific nature of the observed clinical phenotypes.

P-90
The glucose uptake stimulators YM-126414 and YM-138552 are potent uncouplers of the mitochondrial respiration

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The substances YM-138552 (5-chloro-*N*(2-chloro-4-nitrophenyl)-2-hyroxy-3-methylbenz-amide) and YM-126414 (1,3,3-trimethyl-2-(2-phenylaminovinyl)-3*H*-indolium) show a stimulation of glucose uptake in myoblast cell cultures. These results, as well as the observed effect of the compounds on glucose transporter isotype 4 (GLUT4) redistribution, made them interesting candidates for the development of new drugs against type 2 diabetes. We have investigated the effect of these two compounds on the ‘state 4’ respiration of tightly coupled

rat liver mitochondria. Both compounds uncoupled mitochondrial respiration. YM-138552 was as effective (U50: 0.03 ± 0.011 μM) as the well-known uncoupler FCCP (U50: 0.039 ± 0.014 μM) whereas YM-126414 (U50: 24.3 ± 4.2 μM) was nearly as effective as 2,4-dinitophenol (U50: 4.95 ± 1.33 μM). We conclude that the observed stimulation of glucose uptake observed in myoblast cell cultures resulted primarily from the uncoupling activity of the compounds and that the observed redistribution of GLUT4 was a secondary effect, caused by the low energetic state of the cells.

P-91

Genotype–phenotype correlations in a German adPEO family carrying three mutations in POLG

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Objectives: Mutations in the adenine nucleotide translocator (ANT1) and in the twinkle gene cause autosomal dominant progressive external ophthalmoplegia (adPEO), while mutations of DNA polymerase g (POLG) cause both dominant and recessive forms of PEO. Here we report the clinical and genetic findings in a German family with adPEO and mutations in POLG.

Methods: A detailed clinical, histological and genetic testing was performed in the index patient and in other family members.

Results: The index patient presented with ptosis, PEO and exercise intolerance since age 45. She had ragged-red fibers on muscle histology and Southern blot showed multiple mtDNA deletions. There were no mutations in ANT1 and twinkle, but sequencing of POLG revealed three previously described heterozygous missense mutations (T251I, L304R and P587L). The 45-year-old daughter of the index patient was clinically unaffected and carried only the L304R mutation. A 47-year-old nephew was clinically unaffected and had no mutations at all.

Discussion: The P587L mutation has previously been described as the sole mutation in two PEO siblings and in conjunction with the T251I mutation. The exact pathogenic role of these mutations, which are described on the same allele in European families with recessive trait, is not completely understood. L304R has been described as a recessive mutation, and seems to be nonpathogenic in our family, unless the daughter of the index family will develop PEO in the future. We will gain more information on the pathogenic role of these mutations after investigating all members of this adPEO family.

P-92

Somatic and germline mutation in GRIM-19, a dual function gene involved in mitochondrial metabolism and cell death, is linked to mitochondrion-rich (Hürthle cell) tumours of the thyroid

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Oxyphil or Hürthle cell tumours of the thyroid are characterized by their consistent excessive number of mitochondria. A recently discovered gene, GRIM-19, has been found to fulfil two roles within the cell: as a member of the interferon-beta- and retinoic acid-induced pathway of cell death, and as part of the mitochondrial Complex I assembly. In addition, a gene predisposing to thyroid tumours with cell oxyphilia (TCO) has been mapped to chromosome 19p13.2 in one family. A cluster of genes involved in mitochondrial metabolism occurs in this region; one of these is GRIM-19. We have searched for GRIM-19 mutations in a series of 52 thyroid tumours. Somatic missense mutations in GRIM-19 were detected in three of 20 sporadic Hürthle cell carcinomas. A germline mutation was detected in a Hürthle cell papillary carcinoma arising in a multinodular goitre composed of Hürthle cells. No mutations were detected in any of the 20 non-Hürthle cell carcinomas tested, nor in any of 96 blood donor samples. In one of the Hürthle cell papillary carcinomas positive for GRIM-19 mutation, we have also detected a ret/PTC-1 rearrangement. No GRIM-19 mutations were detected in any of the six cases of known familial Hürthle cell tumour tested, so that our results do not support the identification of GRIM-19 as the TCO gene. We propose that mutations in GRIM-19 can be involved in the genesis of sporadic or familial Hürthle cell tumours of the thyroid through the dual function of GRIM-19 in mitochondrial metabolism and cell death.

P-93

Mutation in a nuclear gene involved in mitochondrial protein translation in a patient with early fatal hepato-encephalopathy

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Mitochondrial translation defects are the most common cause of deficiencies in the enzyme complexes of the oxidative phosphorylation (OXPHOS). Although all known mutations of the mitochondrial translation apparatus are encoded by nuclear genes, all known molecular defects associated with impaired mitochondrial translation are due to mutations in mitochondrial DNA. We investigated two siblings with a progressive hepato-encephalopathy, reduced levels of all OXPHOS complexes containing mtDNA-encoded subunits, and a severe defect in mitochondrial translation. Blue Native PAGE analysis of fibroblasts from the index case showed reduced amounts of fully assembled complexes I, III, IV and V, while levels of complex II were normal. With transmitochondrial cybrids it was proved that the assembly defect was of nuclear origin. Using microcell-mediated chromosome transfer, we mapped the defective gene to a region on chromosome 3. With a panel of microsatellite markers, we could narrow down the region to 20cM. A database search for candidate genes in this region identified two genes predicted to code for proteins that are part of the mitochondrial translation machinery. A cDNA sequence analysis revealed a homozygous missense mutation in an evolutionarily conserved residue in one of these genes. The mitochondrial translation defect was complemented with the wild-type human gene proving that the mutation was indeed the cause of the disease. These results define a new class of gene defects underlying OXPHOS disorders.

P-94

Increased free radical production and oxidative damage in transmitochondrial cybrids harbouring the T14487C mutation in mitochondrial DNA

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There is an increasing evidence suggesting that free radical (FR) damage plays a key role in the pathogenesis of cell damage mediated by mtDNA mutations. However, very few systematic studies in controlled experimental models have been performed to assess this hypothesis. We report biochemical data on a cell model of the T14487C mutation in the ND6 gene of mtDNA. Here we show that transmitochondrial cells harbouring homoplasmic levels of the mutation showed an increase in hydrogen peroxide production (0.73 vs. 0.45 nmol H_2O_2/min). Moreover, we also observed an increase of oxidative damage on lipid and protein compounds, as assessed by the measurement of malondialdehyde (0.38 vs. 0.20 nmol MDA/mg prot) and protein carbonyl content (two-fold increase).

These results point out to the possibility that mutations in complex I subunits lead to a significative increase in FR production and a subsequent increase in peroxidative damage on cell lipids and proteins. If free radicals are involved directly or indirectly in the modulation of the clinical phenotype in mitochondrial patients is a question that remains to be clarified. This clarification is important as may result in the development and clinical use of therapeutic strategies addressed to deliver antioxidant agents into dysfunctional mitochondria.

P-95

Accumulation of homoplasmic mtDNA point mutations in erythroblasts isolated from the bone marrow of patients with refractory anemia with ring sideroblasts

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Refractory anemia with ring sideroblasts (RARS) is a hematopoietic stem cell disorder of unknown origin, characterized by excessive iron accumulation in erythroblast's mitochondria. It was hypothesised that mitochondrial iron overload may result from acquired mitochondrial DNA (mtDNA) mutations and subsequent impairment of the respiratory chain complexes function. In our study, we analysed mtDNA sequence of three RARS patients who were selected on the basis of high sideroblasts content in their bone marrow. To analyse the mtDNA sequence of iron storing mitochondria sensitively, we developed new protocols for selective erythroblasts isolation, mtDNA PCR amplification and mtDNA sequencing. Using this approach, we found altogether 84 mtDNA single-nucleotide substitutions. These included 71 already published polymorphisms, five silent mutations in MTATP8, MTND1, MTND2, MTND5, MTCOII

genes and eight novel mutations (two mutations in RNA coding genes MTRNR2 and MTTM and six missense mutations in genes MTATP6, MTATP8, MTND2, MTCYB and MTCOI). With the exception of the mutation G15084T in MTCYB, which was heteroplasmic, all the other detected mutations were homoplasmic. Functional analyses suggested that identified mutations probably do not result in major perturbations of mitochondrial functions and are tolerated (except for heteroplasmic mutation G15084T). We propose a mechanism in which single mtDNA mutation is aquired first. When tolerated, it saturates to homoplasmy and causes minor perturbation of mitochondrial metabolism, which increases mtDNA mutation rate and accelerates generation of additional mutations. These mutations, if tolerated, accumulate to homoplasmy until a threshold level is exceeded and functional impairment results in an ineffective hematopoiesis and defective maturation of erythroblasts.

P-96

POLG and Twinkle mutations causing progressive external ophthalmoplegia and mitochondrial myopathy

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Background: The mendelian forms of progressive external ophthalmoplegia (PEO) are clinically and genetically heterogeneous disorders characterized by accumulation of deletions in mtDNA. They are associated with mutations in three nuclear genes, adenine nucleotide translocator 1 (ANT 1), twinkle helicase (C10ORF2), and the catalytic subunit of polymerase gamma (POLG1), all of them encoding factors involved in mtDNA stability.

Objective and methods: (i) To investigate the frequency and genotype–phenotype correlations of mutations in patients with PEO or mitochondrial myopathy (MM) associated with multiple deletions in mtDNA. Coding regions and exon/intron boundaries of ANT1 and POLG, and mutational hot spot region of C10ORF2 were sequenced. (ii) The study includes 19 patients.

Results: (i) We have identified five novel mutations in POLG1 gene and a novel mutation in Twinkle. (ii) We have partially characterized phenotypically and genetically a series of patients:

Patient 9: PEO. Compound heterozygote for a P587L and the novel missense mutation R853W in POLG1.
Patient 12: PEO. Compound heterozygote for the allele T251I-P587L and the novel mutation M603L in POLG1.
Patient 14: PEO and generalized myopathy. Heterozygous for the novel mutation D1184N in POLG1.
Patient 22: Left gastrocnemius atrophy. Heterozygous for the novel mutation R1146C in POLG1.
Patient 20: adPEO. No deletions in mtDNA. Heterozygous for a novel missense mutation, R357P in Twinkle.

These mutations affect conserved residues and are not detected in 200 healthy control alleles.

Conclusions: We have identified mutations in our patients with a proportion and clinical and genetic heterogeneous manifestations similar to previously reported series.

P-97

Mitochondrial toxicity due to antiviral therapy: decreased mtDNA levels in blood cells of HIV-infected individuals can further decrease or recover depending on the used antiviral drugs

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Mitochondrial dysfunction as a result of depletion of mitochondrial DNA (mtDNA) is one of the determinants of the adverse effects caused by nucleoside analogue (NRTI) treatment in HIV infection. Using a real-time NASBA assay quantifying both mtDNA and nuclear DNA in one tube, we have investigated the effects of some antiviral drugs on the mtDNA content both in vitro and in vivo. In HepG2 cells, we observed an increase in mtDNA copies per cell at low concentrations of d4T (<30 μM) and at all tested concentrations of 3TC and AZT. At higher concentrations of d4T (>30 μM) and all tested concentrations of ddC, a decrease in mtDNA could be observed. Depletion of mtDNA in PBMC due to HIV infection itself was aggravated by therapies containing ddC, and/or ddI, possibly leading to severe adverse effects. In contrast, more recent antiviral therapies, also those containing d4T, resulted in an increase of mtDNA, which is probably due to recovery from HIV infection combined with less toxicity of the antiviral drug combinations itself. Low mitochondrial toxicity may lead to an increase of mtDNA to compensate limited mitochondrial function loss similar to what has been observed in the in vitro experiments. We conclude that the (combination of)

drugs used together with individual determinants are influencing the course of mtDNA during therapy and the development of adverse effects. The consequences of these observations for the prediction of the development of adverse effects, and as such on therapy management, are being investigated in clinical studies.

P-98

Transient infantile hepatic failure, profound mental retardation and white matter abnormalities due to mtDNA deletions

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MtDNA deletions are associated with a number of distinct clinical phenotypes. These include Kearns–Sayre syndrome, Pearson marrow-pancreas syndrome, chronic progressive external ophthalmoplegia and Mitochondrial neuro-gastrointestinal encephalopathy (MNGIE). Gastrointestinal involvement is prominent in Pearson and MNGIE, while white matter changes are especially prominent in MNGIE and KSS. Here we describe a 15-month-old boy who succumbed following multisystem involvement which included: failure to thrive, transient liver and pancreatic insufficiency, lack of any psychomotor development, severe brain atrophy with white matter destruction. Elevated blood lactate and a lactate peak in the basal ganglia (on MRS) were found. Thymidine phosphorylase activity was normal. Muscle, fibroblast and liver respiratory chain enzyme activities were within the normal range. The T8993G mtDNA mutation was excluded. Southern blot analysis revealed multiple deletions. These deletions were restricted to the liver.

To the best of our knowledge, this infantile devastating clinical presentation with a combination of gastrointestinal and white matter involvement has not been previously described in association with mtDNA deletions, and thus represents a further expansion of the spectrum of mitochondrial DNA deletion syndromes.

P-99

Mitochondrial and nuclear genome responses in cells with arrested transcription and translation

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Many mutations found in the mitochondrial and nuclear genome cause impairment of mitochondrial translation or replication/translation systems (e.g. mutations in mt-tRNA genes, depletions). The conditions existing in cells with such mutations can be simulated by treatment with exogenous compounds. Thamphenicol and ethidium bromide are commonly used inhibitors of mitochondrial translation and transcription, respectively. We present changes in steady-state levels of mitochondrial transcripts and of mitochondrial proteins encoded by nuclear genes in cells treated with thamphenicol and ethidium bromide. Alterations in activity of the glycolytic pathway and respiratory chain content are also presented.

P-100

Characterisation of undifferentiated and differentiated RD-cybrids, harbouring 3243 MELAS mutation

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Human cell lines harbouring MELAS 99% A3243G mutant mtDNA in rhabdomyosarcoma background (MELAS RD-cybrids) have established and induced to differentiate after TPA treatment (aneural); further differentiation has been also obtained by innervation of myotubes, using transverse sections of embryonic spinal cord with dorsal root ganglia attached (neural).

Mitochondrial shape and distribution in MELAS and WT RD-cybrids were studied in undifferentiated, aneural and neural cybrids.

In contrast to undifferentiated cybrids, neural MELAS RD-cybrids displayed altered mitochondrial morphology, compared to control RD-cybrids. Neural WT myotubes had threadlike mitochondria distributed throughout the myotubes, whereas mitochondria of MELAS myotubes clustered around the nucleus. Mitochondrial distribution, expressed as pixel area, was significantly reduced in MELAS RD-cybrids compared to controls. Perinuclear clustering of mitochondria was confirmed by TEM analysis. At high magnification MELAS mitochondria were larger, swollen compared to WT, with abnormal tubular cristae. The mitochondrial matrix contained dense grains and crystals; a difference of undifferentiated MELAS mitochondria that were similar to control. Treatment with antioxidant *N*-acetyl-cysteine (0.5 mM) (NAC) restored the threadlike shape and normal distribution on mitochondria in differentiated MELAS RD-cybrids. Also ultra-

structural analysis revealed a decrease of dense grains and normal cristae in mitochondria of MELAS differentiated cells after NAC treatment. Aneural MELAS RD-cybrids were similar to neural myotubes. In conclusion, differentiated, but not undifferentiated, MELAS RD-cybrids show mitochondria perturbation that is ameliorated by treatment with antioxidant NAC.

P-101
Frataxin interacts with proteins involved in iron–sulfur cluster biogenesis and repair in human cells

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Microarray analysis in human cells have supported a role for frataxin in iron–sulfur biogenesis and repair (Tan et al., 2003). To identify frataxin's interactors, we have carried out analyses by co-immunoprecipitation, mass spectrometry, and blue native gel electrophoresis. We observed that anti-frataxin antibody co-immunoprecipiated the human ISC-U homolog, consistent with our observation that ISC-U levels are decreased in cells of patients with Friedreich's ataxia. In addition, we observe co-immunoprecipitation of frataxin with rhodanese, which repairs mitochondrial ISCs, and strong correlation of frataxin with rhodanese expression. Mass spectrometry of other mitochondrial proteins immunoprecipitated by anti-frataxin antibody included dnaK, which interacts with ISC proteins during their biogenesis. However, we observed no interaction of frataxin with ISC-S, the sulfur donor in ISC biogenesis, by co-immunoprecipitation, and little alteration of ISC-S levels in human cells. Furthermore, we did not observe any co-migration of frataxin and HSC20, the mammalian homolog of the bacterial HSC20. Thus, although frataxin interacts with multiple proteins thought to be essential for mitochondrial ISC biogenesis and repair (ISC-U, rhodanese, dnaK), consistent with a function of frataxin as an ISC protein co-chaperone, it does not appear to interact with ISC-S and HSC20 homologs. As the presumptive consequence of this ISC defect, we observe decreased activity of the ISC-dependent enzymes, increased mitochondrial free iron, increased level of mitochondrial superoxide and peroxide, and increased activity of the antioxidant enzyme glutathione peroxidase and increased amount of GSSG in mutant cells; these data fit into a unified model of FRDA pathophysiology which will be presented.

P-102
Mitochondrial abnormalities on peripheral blood mononuclear cells of HIV infected patients

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Objective: Depletion of mitochondrial DNA (mtDNA) has been considered a marker of mitochondrial toxicity associated with antiretroviral therapy, although HIV infection itself could play a role in this effect. We determine the mtDNA content in peripheral blood mononuclear cells (PBMC) in naive HIV-infected patients and its potential consequences in mitochondrial respiratory chain (MRC) function.

Design: A prospective, cross-sectional study with cases and healthy controls.

Methods: We measured: (1) the quantity of mtDNA by real-time PCR; (2) the enzyme activities of complexes II, III and IV of the MRC, citrate synthase (CS) and glycerol-3-phosphate dehydrogenase (G3PD) by spectrophotometry; and (3) membrane lipid peroxidation by fluorimetry, in PBMC of 25 asymptomatic HIV-infected patients who had never received antiviral therapy and of 25 healthy controls.

Results: In the HIV-infected group we found a 32% decrease of mtDNA ($P = 0.002$) and a decline in the enzyme activities of complex II (41% decrease, $P < 0.001$), complex III (38% decrease, $P < 0.001$) and complex IV (19% decrease, $P = 0.001$) compared to controls. Both mtDNA depletion and decreased MRC enzyme activities remained significant, even when assessed per organelle (mitochondria). CS activity did not differ between the two groups. G3PD activity was found to be decreased by 22% ($P < 0.001$) in the HIV-infected group. Lipid peroxidation of PBMC was increased in HIV-infected patients compared to controls ($P = 0.007$).

Conclusions: HIV infection is associated with mtDNA depletion and extensive MRC disturbances not only limited to complexes encoded by mtDNA. Therefore, other mechanisms different from mtDNA depletion, like apoptosis, could be responsible for such mitochondrial damage.

P-103
Molecular characterization of a mouse cell line with a COX10 gene knockout

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Cytochrome *c* oxidase (COX) has been associated with multiple neuromuscular and neurodegenerative diseases. COX biosynthesis depends on a large number of integral subunits as well as assembly factors. COX10, an assembly factor, is a protoheme:heme *O* farnesyl transferase involved in heme a synthesis. COX10 has been found to be mutated in some patients with COX deficiency.

We established a fibroblast cell line by infecting a primary cell culture from a mouse homozygous for the floxed COX10 gene (exon 6) with a retrovirus expressing E6/E7. To generate a cell culture model for COX deficiency, this cell line was transfected with a plasmid expressing Cre recombinase. Clones obtained were analyzed for exon 6 deletion and for COX activity. Total cytochrome analysis from mitochondrial preparations reveals the absence of only cyt aa3 in the knockout clones. KO cells had undetectable levels of COX I by immunocytochemistry and Western blot. KO clones also showed decreased complex I + III and II + III enzyme activities. Analysis of respiratory complexes by blue native gels also suggests that the levels of fully assembled complex I and complex III are moderately decreased in the KO cells. We were unable to detect formation of fully assembled or subcomplex assemblies of COX as described in patients with COX10 mutations. We were unable to detect any oxidative damage (protein oxidation, H_2O_2 and super oxide formation) in COX-deficient cells.

This cell culture model of COX deficiency should be instrumental in helping us understand the pathophysiology of COX deficiencies as well as in developing treatments.

P-104
Functional and diagnostic consequences of a novel mitochondrial tRNA mutation co-segregating with a D-loop polymorphism

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We report on the biochemical and genetic analysis of a 28-year-old female patient presenting with chronic progressive external ophthalmoplegia (CPEO). In a muscle biopsy showing multiple ragged red fibers, we identified a novel heteroplasmic G12276A mutation, residing in the mitochondrial tRNA Leu(CUN) gene, as well as a previously described heteroplasmic D-loop polymorphism (A185G). Both mutations were also present in fibroblasts, buccal mucosa and blood, although at much lower levels than in skeletal muscle. Northern blot analysis demonstrated a 50% reduction of Leu (CUN) tRNA in skeletal muscle in comparison to controls, indicating the low stability of mutated tRNA due to DHU-stem mispairing. This is most likely the mechanism how the G12278A mutation leads to general failure of mitochondrial translation. As a consequence, individual muscle fibers harbouring G12276A mutation loads higher than 80% showed severe decrease in cytochrome *c* oxidase (COX) activity. This decrease of COX activity was unexpectedly also seen in fibers showing a high degree of A185G heteroplasmy. Comparing the distribution of G12276A and A185G mutations, we found that three allelic combinations were present in skeletal muscle. Our single fiber analysis demonstrated a co-segregation of the mutant 12276A allele with the wild-type 185G allele, which readily explains the pseudo-correlation of the neutral 185G D-loop polymorphism with the pathological biochemical phenotype. Beyond the identification of the pathomechanism of a novel mutation in the mitochondrial tRNA Leu(CUN) gene causing CPEO, our study highlights the importance of whole mtDNA genetic analysis in assessing the pathogenic relevance of individual heteroplasmic mtDNA point mutations.

P-105
Sensory ataxic neuropathy due to a novel C10Orf2 (Twinkle) gene mutation with germline mosaicism

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Autosomal progressive external ophthalmoplegia (PEO) typically presents with ptosis and weakness of the extraocular muscles due to the accumulation of multiple mitochondrial DNA (mtDNA) deletions in skeletal muscle. Many patients develop with limb weakness and some have multisystemic involvement including hearing loss and psychiatric abnormalities. Mutations in three nuclear genes (POLG1, C10Orf2 which codes for the protein Twinkle, and ANT1) are found in ~ 70% of families with dominant PEO, and ~ 1/3 of sporadic cases of PEO with multiple mtDNA deletions. We sequenced ANT1, C10Orf2, and POLG1 cohort of 25 sporadic English and German patients with PEO and multiple deletions and identified a novel C10Orf2 mutation in one family with germline mosaicism. One

sibling presented with the clinical triad of sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO), a phenotype previously associated with the POLG1 gene, illustrating the phenotypic overlap in autosomal PEO.

P-106
MtDNA complementation and recombination in human cells harboring pathogenic mutations in polypeptide-coding genes

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Complementation between mitochondrial (mt) DNA molecules may represent an important line of defense against the potentially detrimental effect of pathogenic mtDNA mutations. Functional complementation between mtDNAs harboring different tRNA mutations or deletions has been previously demonstrated in mammalian cells. Therefore, in order to assess whether complementation can occur between mtDNAs harboring mutations in protein-coding genes, we have generated hybrids by fusion of two human cybrid cell lines each containing homoplasmic levels of a distinct mtDNA mutation in a protein-coding gene. One mutation resulted in the loss of subunit 1 of cytochrome oxidase (COX1), the other resulted in loss of cytochrome B. Individually these mutations caused complete lack of mitochondrial oxidative phosphorylation. We showed that, in the COX1–Cytochrome B hybrids, oxidative phosphorylation was recovered suggesting that functional complementation had indeed occurred. In addition, we found evidence that, in the hybrids, the two species of mtDNA had undergone genetic recombination resulting in novel mtDNA molecules. Although intermolecular mtDNA recombination is known to occur in yeast and plants, its existence in human mitochondria is still controversial. To our knowledge, this is the first demonstration that mtDNA recombination among mutant mtDNAs can occur in mammalian cells.

P-107
MtDNA deletions and not point mutations cause mitochondrial myopathy in PEO with POLG1 mutations

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Objective: To investigate whether mtDNA point mutations, directly or indirectly, are involved in the pathogenesis of mitochondrial myopathy in progressive external ophthalmoplegia (PEO) associated with POLG1 mutations. POLG1 encodes the catalytic alpha subunit of polymerase gamma and is the only polymerase known to be involved in mtDNA replication. It has two functionally different domains, one polymerase domain and one exonuclease domain with proofreading activity.

Methods: Muscle biopsy specimens from patients with POLG1 mutations, affecting either the exonuclease or the polymerase domain, were investigated. Single cytochrome *c* oxidase (COX)-deficient muscle fibers were dissected and screened for clonally expanded mtDNA point mutations by a sensitive denaturing gradient gel electrophoresis analysis, in which three different regions of mtDNA including five different tRNA genes were investigated. To screen for randomly distributed mtDNA point mutations in muscle homogenate, two regions of mtDNA including deletion breakpoints were investigated by high-fidelity PCR, followed by cloning and sequencing. Large-scale mtDNA deletions were investigated by long-range PCR analysis.

Results: All patients but not controls harboured multiple mtDNA deletions. No point mutations were identified in single COX-deficient muscle fibers. Cloning and sequencing of muscle homogenate identified occasional point mutations at very low frequency in some of the patients.

Conclusions: mtDNA point mutations appear not to be directly or indirectly involved in the pathogenesis of mitochondrial disease in patients with different POLG1 mutations. These findings suggest that it is the polymerase and not the proofreading activity of POLG1 that is disturbed in PEO and cause multiple mtDNA deletions.

P-108
Dominant-negative effect of specific mouse genes in a human nuclear and mitochondrial background

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Numerous strategies for the identification of a genetic defect in patients with autosomal recessive mitochondrial disorders have been used, including linkage analysis, overexpression of candidate cDNAs, and functional complementation by microcell-mediated chromosomal transfer. Each of these strategies has its limitations. Linkage analysis cannot be used when family size is small, which is the situation in the majority of cases. Chromosome transfer is the most generally appreciable method; however, deletion mapping is complicated by the low rate of informative markers after transfer.

We reasoned that chromosomal transfer might be made more efficient if mouse chromosomes were used instead of human chromosomes, as all chromosomes transferred would be informative. To test this, we first fused patient and control cell lines with mouse rho0 cells. The defect in patient cells was not complemented; in fact, the fusion of a control human fibroblast line or a human osteosarcoma line with mouse rho0 cells resulted in severely decreased activities and protein levels of oxidative phosphorylation enzymes. The transfer of individual mouse chromosomes into patient cells partially rescued the biochemical defect in several patients. Alongside the rescue, we observed in some cells a strong dominant negative effect of certain mouse chromosomes on mitochondrial translation, activity and protein levels of complex I and/or complex IV.

These data suggest a dominant negative effect of specific mouse genes when present in a human nuclear and mitochondrial background. We are currently mapping these genes. Identifying the genes may provide some insight into the regulation of oxidative phosphorylation in higher vertebrates.

P-109

Clinical, biochemical and molecular analyses in 177 children with COX deficiency

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We present clinical, biochemical and molecular analyses in 177 children with cytochrome *c* oxidase (COX) deficiency from 140 families from Poland, Czech and Slovak Republic with 53 million inhabitants. *Methods*: Activities of respiratory chain complexes (RC) were measured in fibroblasts or muscle mitochondria spectrophotometrically. Amount and composition of RC were studied by 2D-PAGE. SURF1 and SCO2 genes and mtDNA mutations were analysed by cyclic sequencing and PCR-RFLP. *Results*: Clinical symptoms started before the age of 3 months in 39% of children and between 3 and 18 months in 40%. The most frequent symptoms were failure to thrive (67%), neuronal impairment (90%) and cardiomyopathy (23%). Sixty-seven percent of children died in early childhood. Increased level of B-lactate, CSF-lactate, B-alanine and CSF-alanine was found in 85%, 81%, 65% and 73% of investigated patients. Isolated COX deficiency was found in 99 children, combined COX deficiency in 78 children. Mutations in SURF1 gene were present in 46 patients (841delCT mutation in 72% of investigated alleles) and mutations in SCO2 gene in 8 children (G1541A mutation in 80%). MtDNA mutations were found in 18 children (mtDNA deletion or depletion 8 ×, A3243G 6 ×, A8344G, A8348G, G8364A and del9205TA once). *Conclusion*: COX deficiency represents a heterogeneous group of diseases except for children with Leigh syndrome and SURF1 mutations. Mutations 841delCT in SURF1 gene and G1541A in SCO2 gene are prevalent, at least in our Slavonic population. Detailed characterisation of COX at the protein and molecular level is necessary for genetic counselling and prenatal diagnosis in affected families.

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P-110

Clinical similarities and differences of four children with mitochondrial DNA deletions

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Single deletions of mitochondrial DNA (del mtDNA) are associated with a large range of various clinical features. Clusters of symptoms known as Kearns–Sayre syndrome and chronic progressive ophthalmoplegia were described in adults and Pearson marrow-pancreas syndrome in children. A set of nonspecific symptoms and signs differs from patient to patient.

The aim of our study is to provide a description of clinical course in four children with single mtDNA deletions. Failure to thrive, stunded growth (without GH deficiency) and fatigability began from infantile period to the age of 2. During the following years multiorgan involvement developed. Hypoparathyroidism, diabetes mellitus and suprarenal insufficiency appeared at the age of 7 and the disorders had nontypical, strange course. Heart conductive abnormalities were present in three cases from late childhood to adolescence. Pearson-like diarrhea episode was seen only once. Macrocytic anaemia was found in two out of four cases. Kidney leakage of magnesium (evoking hypoparathyroidism) and sodium (imitating hypocorticism) as well as urinary phosphate and potassium loss were frequently observed. Serum and cerebrospinal fluid lactate levels were elevated. Delay of the diagnosis was from 2 to 14 years.

In our opinion, a major condition to improve detection of children with mitochondrial disorders is to measure lactate levels in all available biological specimens, routinely. Early referral to a diagnostic metabolic (mitochondrial) centre is

also important. Atypical or ''strange'' course of progressive chronic disorder, when associated with lactic acidaemia, should especially alert not only haematologists but also paediatric endocrinologists and nephrologists.

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P-111

Inhibition of mitochondrial Na^+/Ca^{2+} exchange restores stimulus-induced ATP production and Ca^{2+} handling in human complex I deficiency

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Human mitochondrial complex I (NADH:ubiquinone oxidoreductase) of the oxidative phosphorylation (OXPHOS) system is a multi-protein assembly comprising both nuclear- and mitochondrially encoded subunits. Deficiency of this complex is associated with numerous clinical syndromes ranging from highly progressive, often early lethal encephalopathies, of which Leigh disease is the most frequent, to neurodegenerative disorders in adult life, including Leber's hereditary optic neuropathy and Parkinson disease. We show here that the cytosolic Ca^{2+} signal in response to hormonal stimulation with bradykinin was impaired in skin fibroblasts from children in the age of 0–5 years with an isolated complex I deficiency caused by mutations in nuclear-encoded structural subunits of the complex. Inhibition of mitochondrial Na^+/Ca^{2+} exchange by the benzothiazepine CGP37157 completely restored the aberrant cytosolic Ca^{2+} signal. This effect of the inhibitor was paralleled by complete restoration of the bradykinin-induced increases in mitochondrial Ca^{2+} concentration and ensuing ATP production. Thus, impaired mitochondrial Ca^{2+} accumulation during cytosolic Ca^{2+} mobilization is a major consequence of human complex I deficiency, a finding which may provide the basis for the development of new therapeutic approaches to this disorder.

P-112

Late-onset disease model for adPEO-Twinkle

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Autosomal dominant progressive external ophthalmoplegia (adPEO) is a mitochondrial disease of adult onset, characterized by ophthalmoplegia, ptosis and exercise intolerance, as well as a myriad of additional family-specific symptoms ranging from major depression, parkinsonism, and secondary amenorrhea, to peripheral neuropathy. The muscle samples of the patients show that 3–10% of muscle fibers are COX-negative ragged red fibers and contain multiple deletions of mitochondrial DNA (mtDNA). We and others have characterized three PEO-genes, which all can be thought to affect mitochondrial DNA maintenance: Twinkle, a mitochondrial helicase, mtDNA polymerase gamma and adenine nucleotide translocator 1.

To study Twinkle-PEO pathogenesis, we created nine transgenic mouse lines, which overexpress wild-type Twinkle or two different patient mutations under ubiquitous beta-actin promoter. The mutant mice develop normally, with no detectable differences compared to the wild-type overexpressor until 1 year of age, when they manifest a late-onset mitochondrial myopathy. We describe detailed characterization of these novel mice, with age-related accumulative dysfunction of the mitochondrial respiratory chain.

P-113

Investigation of the genetic basis of isolated Complex I deficiency

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This study aims to investigate the genetic basis of isolated autosomal recessive Complex I deficiency in seven patients with apparent defects in the assembly of the mature enzyme. To determine the number of genetic complementation groups, the patient cell lines were fused to one another in a pairwise fashion. In all cases except one (siblings), the fusion of two patient cell lines resulted in the rescue of the Complex I defect. This experiment showed that there are six different disease-causing genes in this group of patients.

Mutations in eight of the nuclear-encoded structural subunits of the enzyme have previously been described in patients with Complex I deficiency, (NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFV1, and NDUFV2). Retroviral expression vectors, containing the above Complex I structural subunits, are being transduced into our patient cell lines to test whether overexpression of these subunits will rescue the defects. To date, overexpression of NDUFS4 was found to rescue the defect in one patient. A mutation in this gene was then confirmed by sequencing.

Finally, in order to try to discover other genes involved in Complex I deficiency, microcell-mediated chromosome transfer was performed on one patient using a mouse donor line. The defect in this patient was rescued by the presence of mouse chromosomes, and genotyping studies are being carried out to try to identify the chromosome involved.

P-114
Ischemic stroke and MELAS syndrome in young adults

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Background and purpose: A rare cause of ischemic stroke in young adults is the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). Previous publications suggest that around 10% of patients with occipital infarction (age <45 at diagnosis) harbour the typical mitochondrial MELAS mutation and around 2% of all premature strokes (age <30 at diagnosis) were diagnosed with mitochondrial disease. The purpose of our study was to examine the frequency of the typical A3243G mutation in young patients (age <45 at diagnosis) presenting with ischemic stroke and to find out whether it may reasonable to screen the patients for this mutation routinely.

Methods and results: Since December 2001, altogether 75 patients were referred to our hospital with acute cerebral ischemia (12 patients with transitory ischemic attack, 63 patients with manifest stroke). They underwent a neurological examination and standard diagnostic procedures, including blood examination, Doppler and duplex sonography, cardiological and neuroradiological examination. Additionally mitochondrial DNA was analyzed for the A3243G MELAS mutation. In one patient we could identify this mutation, thereby the patient showed most of the clinical characteristics with lactic acidosis, seizures, sensoneurinal deafness, diabetes mellitus and short stature.

Conclusions: MELAS syndrome is a rare cause for ischemic stroke in young adults. Within 75 patients we identified one with the typical clinical characteristics and the mitochondrial A3243G mutation. In patients without the typical clinical symptoms of the disease, we could not find the mitochondrial mutation.

P-115
Quinone analogues regulate mitochondrial substrate competitive oxidation

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Quinone derivatives are among the rare compounds successfully used as therapeutic reagents to fight mitochondrial diseases. However, their beneficial effect appears to depend on their side chain which presumably governs their interaction with the respiratory chain. The effect of four quinone derivatives was comparatively studied on NADH- and succinate-competitive oxidation by a sub-mitochondrial fraction. Under our experimental conditions, the less hydrophobic derivatives (menadione, duroquinol) poorly affected electron flow from either NADH or succinate to oxygen, yet readily diverting electrons from isolated complex I. This latter effect was abolished by succinate addition. More hydrophobic derivatives (idebenone, decylubiquinone) stimulated oxygen uptake from succinate. But while NADH oxidation was slightly inhibited by idebenone, it was somewhat increased by decylubiquinone. As a result, idebenone strongly favoured succinate over NADH oxidation. This study therefore suggests that any therapeutic use of quinone analogues should take into account their specific effect on each respiratory chain dehydrogenase.

P-116
Developing a mouse model of the mitochondrial NARP syndrome using AAV-ribozymes for ATP6

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Neuropathy Ataxia and Retinitis Pigmentosa (NARP) is caused by point mutations in the mitochondrial gene ATP6 and leads to various types of retinopathies as well as dementia and skeletal muscle weakness. There are currently no animal models of this disease, so we are developing a mouse model using an AAV-vectored ribozyme imported into the mitochondria to disrupt the ATP6 mRNA. The ribozyme is tagged on the end of the 5S rRNA for targeting to the mitochondria.

In cultured mouse cells expressing our ribozyme, we have shown a reduction in the target mRNA by quantitative RT-PCR, a defect in ATP synthesis in permeabilized cells, and a growth defect in galactose media. We have also looked at other mitochondrial genes and saw a reduction in COX3 and ND4. These cells were also found to have increased resistance to cyanide, an indication of reduced cytochrome oxidase. We are currently trying to rescue this defect with a nuclear-encoded version of mouse ATP6 targeted to the mitochondria. If we can rescue the defect with a single protein, it would suggest that the reduction of other mitochondrial mRNA is a secondary effect. Mice have

also been injected sub-retinally with the AAV-vectored ribozyme and are being monitored by electroretinogram (ERG.)

P-117
Mitochondrial DNA alterations in post-Chernobyl thyroid tumours

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Following Chernobyl accident, a dramatic increase in childhood thyroid cancers was observed. Little is known about the effects of radiation over the mitochondrial genome (mtDNA). The aim of this work was to evaluate the effect of radiation on mtDNA and the role of mtDNA alterations in radiation-induced thyroid tumours. We studied DNA from tumour tissue and adjacent thyroid parenchyma in eight follicular thyroid adenomas (FA) and 15 papillary thyroid carcinomas (PTC), from which DNA from peripheral blood leukocytes was also available. We analysed the d-loop region in all cases, and the 13 mtDNA genes coding for proteins of the oxidative phosphorylation system in five PTC. We detected d-loop instability in one of eight FA and in nine of 15 PTC. MtDNA point mutations were also detected in all the five PTC. In the five PTC, mtDNA mutations identical to those detected in the tumours were detected in the adjacent thyroid parenchyma but not in the respective blood samples. The comparison of this series with a similar series of sporadic (nonirradiated) thyroid tumours disclosed a much higher number of mtDNA alterations in the former. Our results support the conclusion that radiation causes mtDNA mutations and show that it is necessary to compare both tumour and non-tumoural findings in the thyroid with a relatively non-exposed tissue such as the blood. A larger series and a thorough study of irradiated thyroids without tumours are necessary to evaluate whether or not mtDNA mutations are involved in the causation of post-Chernobyl thyroid tumours.

P-118
Transport dysfunction in mitochondrial diseases: mutational analysis and transcription factors of hTomm and hTimm genes

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To understand the molecular bases of mitochondrial diseases that could be due to defects in the mitochondrial transport machinery, we carry out a comprehensive analysis of alterations in the subunits of the Translocase Outer and Inner Mitochondrial Membrane, TOMM and TIMM, complexes. The mutational analysis includes exons, splice junctions and promoter regions of hTomm and hTimm genes in 288 DNA samples from patients. We report the finding of several novel heterozygous DNA alterations including frameshift and missense mutations. In addition, Nuclear Respiratory Factors (NRFs) are involved in governing nucleo-mitochondrial interactions promoting the expression of genes encoding respiratory subunits and key components of the mtDNA transcription and replication machinery. TOMM70 plays a major role as a receptor of hydrophobic preproteins targeted to the mitochondria. TOMM20 recognizes preproteins containing N-terminal as well as internal targeting signals. Analyses of the promoter regions of these human genes reveal the presence of putative NRF-1 and NRF-2 binding sites. To characterize the activity of the Tomm70 and Tomm20 promoter, we designed constructs containing different promoter regions that were cloned in the pGL3-basic vector and assayed for functionality after transient transfections by using a luciferase reporter assay system. Mobility shift assays and super-shift experiments using antibodies against NRF-2a were also performed. Our results provide evidence for the role of NRFs as transcription factors which are involved in the regulation of these genes. This supports the idea that function of NRFs in nucleo-mitochondrial coordination could be extended to major receptors of the mitochondrial protein import machinery.

P-119
Endurance training in patients with single, large-scale mtDNA deletions: molecular genetic studies

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Previous studies have demonstrated that aerobic training can increase work and oxidative capacity in patients with pathogenic mitochondrial DNA (mtDNA) mutations. Although the mechanisms underlying physiological improvement are not fully understood, they are attributed to training-induced increases in mitochondrial copy number which, in most patients, resulted in increased levels of rate-limiting enzymes (i.e. those affected by the mutation). While this finding suggests an increase in absolute levels of wild-type mtDNA, increases in the relative proportion of mutant genomes were detected. Despite biochemical and physiological improvements supporting the application of endurance training in the management of patients with mtDNA disorders, the expansion of mutant mtDNA raises potential problems and, at present, we are unable to give clear advice to our patients.

To explore these issues, seven patients with well-characterised, single mtDNA deletions were subjected to 14 weeks of endurance training followed by 14 weeks of either maintenance training at similar intensity or a deconditioning phase; physiological evaluation and muscle biopsy were performed at baseline, 14 and 28 weeks. For each of the biopsies, we present the following data:

[1] Activities of respiratory chain complexes and the matrix marker citrate synthase in muscle homogenates
[2] Mitochondrial histochemistry evaluating the percentage of respiratory-deficient fibres
[3] Southern blot analysis investigating changes in mtDNA heteroplasmy in muscle homogenates
[4] Real-time PCR analysis of cytochrome *c* oxidase (COX)-deficient and COX-positive fibres to establish whether changes in the level of mtDNA deletion or mtDNA copy number within individual fibres play a crucial role in determining enzyme activity.

P-120
Leber's Hereditary Optic Neuropathy mutations in Poland

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Leber's Herereditary Optic Nauropathy (LHON) is caused by various point mutations in mitochondrial DNA. Most of them are located in complex I genes. Three mutations (G11778A, G3460A, T14484C) are responsible for about 90% of Leber's cases. The G11778A mutation is diagnosed in about 69% of cases in Caucasian population. Besides these common mutations there are multiple rare mutations causing the disease. There seems to be a ''hot spot'' in the ND6 gene because there are already five mutations designed to this locus.

In multiple studies the correlation between mitochondrial haplogroup J and G11778A, T14484C mutations was observed.

LHON is molecularly diagnosed in Poland from 2000. We show the results of the common mutation screening and haplogroup analysis of Polish patients with LHON. No correlation between G11778A and haplogroup J was observed. There are some data suggesting the correlation between T14484C and J haplogroup, but the sample is too small to make any statements.

In all the patients negative for three common mutations we also looked for new mutations in ND6 gene by SSCP and sequencing.

P-121
Genotype–phenotype correlation in children with mutations in SCO2 and a novel mutation 1518delA

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Cytochrome *c* oxidase (COX) deficiency represents one of the most common mitochondrial disorders in childhood. Sco2 protein, encoded by nuclear SCO2 gene, presumably affects provision of Cu+ to CuA site of MTCO2. We present genotype–phenotype correlation in seven children with isolated COX deficiency and mutations in SCO2 gene. *Results*: Molecular analyses revealed five children homozygous for missense mutation G1541A (E140K), two other compound heterozygous for mutation G1541A and nonsense mutation C1280T (Q53X) or novel frame-shift mutation 1518delA, which disrupts the reading frame of mRNA and gives rise to a truncated protein. In homozygous patients, the clinical symptoms including inspiratory stridor, hypotonia, encephalopathy and progressive respiratory insufficiency developed between the third and sixth month of life. The onset of the disease started in both heterozygotes at birth. Severe hypertrophic cardiomyopathy was observed in the child with mutations C1280T/G1541A and fatal neonatal encephalopathy with cardiomyopathy in the child with mutations 1518delA/G1541A. In homozygous patients, the maximum of histopathological changes was found in the brain with neuronal degeneration and infrequent increase in the amount of neuronal mitochondria. No neurogenic atrophy of skeletal muscles, axonal degeneration or myelin breakdown in skin nerves was observed. No substantial changes were found in the heart. On the contrary, prominent hypertropic cardiomyopathy without significant changes in other organs was found in the child with mutation C1280T. *Conclusion*: The disease was fatal in all our children with COX deficiency and SCO2 mutations, but the course of the

disease remarkably differed depending on the type of mutations. Supported by GACR-303/03/D132.

P-122
Regulation of cytochrome *c* release via mitochondrial NO-cGMP pathway in rat cardiac myocytes

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Although the existence of cardiac mitochondrial cGMP has been reported (Kimura and Murad, 1974), its physiological property has been unknown. Recently, we have demonstrated an increase in cardiac mitochondrial cGMP level induced by nitric oxide (NO) donors. Then, we investigated to clarify whether the increase in mitochondrial cGMP by NO induces cytochrome *c* release from mitochondria. Mitochondria were prepared from left ventricular myocytes of male Wistar rats (350–400 g) and were incubated in high-K medium (150 mM) at 30 °C. SNAP, an NO donor, accelerated cytochrome *c* release from mitochondria, which was significantly inhibited by ODQ, a guanylyl cyclase inhibitor, and oxyhemoglobin, an NO scavenger. A membrane-permeable cGMP analogue, 8-Br-cGMP, also increased cytochrome *c* release. However, SNAP and 8-Br-cGMP neither induced mitochondrial swelling nor depolarized the mitochondrial membrane potential. We further confirmed that NO donor induced apoptosis in the cultured cardiomyocytes from adult rats, which was significantly inhibited in the presence of ODQ. These results suggest that mitochondrial NO-cGMP pathway in cardiac myocytes causes cytochrome *c* release from mitochondria independent of mitochondrial permeability transition pore, resulting in induction of apoptosis. Further investigations are necessary to clear the mechanism of cytochrome *c* release via mitochondrial NO-cGMP pathway in detail.

P-123
A potential disease mechanism for mutant SOD1-associated ALS

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Transgenic mice overexpressing the human SOD1 gene containing a Gly to Ala substitution at amino acid position 93 (G93A-SOD1) were used to examine the mitochondrial involvement in the pathogenesis of ALS. We observed a decrease in mitochondrial respiration in brain and spinal cord of the G93A-SOD1 mice. This decrease was significant at the last step of the respiratory chain (cytochrome *c* oxidase), and it was not observed in transgenic wild-type-SOD1 and non-transgenic mice. By adding exogenous cytochrome *c* to brain mitoplasts, the defect was abolished. Western blot studies showed that there was an increased cytochrome *c* dissociation from the inner mitochondrial membrane. Dissociation of cytochrome *c* from the inner membrane has been associated with oxidative damage to mitochondrial lipids, a process that can be reversed by SOD1/catalase treatment. Preliminary in vitro assays showed that the G93A SOD1 had a lower capacity than the wt SOD1 to revert the cytochrome *c* dissociation from the inner mitochondrial membrane. Lipid peroxidation studies showed that mitochondrial membranes in G93A SOD1 mice were three to eight times higher than in total homogenates when compared to control mice. We propose a mechanism by which mutant SOD1 predisposes neurons to cytochrome *c* release and apoptotic death by increasing the dissociation of cytochrome *c* from the inner mitochondrial membrane.

P-124
Mitochondrial DNA (mtDNA) levels vary in subcutaneous adipose tissue from HIV-infected patients

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Recently several studies have shown decreases in mtDNA levels in subcutaneous adipose thigh tissues from lipoatrophic HIV-infected patients taking nucleoside reverse transcriptase inhibitors (NRTIs) [JAIDS 30 (2002) 271; JAIDS 29 (2002) 117; AIDS 15 (2001) 1801]. Subcutaneous adipose tissue can be obtained from different sites and mtDNA copies/cell have not been compared until this study. Peripheral adipose tissues were obtained from neck, abdomen, and thigh of seven HIV-seropositive patients treated with NRTIs-containing highly active antiretroviral therapy (HAART) with lipoatrophy for >6 months, seven HIV-infected patients treated with NRTIs-containing HAART with no lipoatrophy, five HIV-infected patients naïve to HAART, and five HIV-seronegative participants. Total DNA was isolated and integrity examined by agarose gel electrophoresis. Intact DNA was assayed for mtDNA copies/cell by real-time PCR using primers for the mitochondrial NADH dehydrogenase 2 and the nuclear Fas genes. MtDNA values were highest in seronegative patients' neck fat, 976 + 292, and abdomens, 790 + 292, and lowest in thighs, 435 + 63 ($P < 0.05$). MtDNA levels were decreased 40–70% in seropositive patients taking NRTIs compared to seropositive naïve and seronegative participants. Depletion was most severe in the neck and abdomen compared to thigh. These studies dem-

onstrate that mtDNA levels vary in different subcutaneous adipose depots suggesting metabolic differences.

P-125
Development and optimization of human MitoChip

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Analysis of gene expression profiles became a powerful tool in the cloning of disease genes and provides functional insight into normal physiology and disease pathogenesis.

We decided to develop MitoChip, an oligonucleotide microarray, which will interrogate ultimately expression of all ≫800 human genes involved in mitochondria biogenesis, maintenance, and metabolism.

To test all the aspects of MitoChip fabrication and analysis, we, in a first round, selected 200 mitochondrial genes involved in energy metabolism, oxidative stress and fatty acid oxidation. For each gene we designed a unique 40-bp-long 5′-amino-modified oligonucleotide. We optimized methods for oligonucleotide attachment on poly-L-lysine-coated glass slides, methods of cDNA labeling and hybridization conditions and, currently, we tested gene expression profiles in various control cell cultures and human tissues.

We believe that MitoChip will become an important analytical approach for various mitochondria related studies.

P-126
Severe defect of complex I-dependent ATP synthesis shapes the mitochondria-driven path of apoptotic cell death in Leber's Hereditary Optic Neuropathy

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We recently reported [J. Biol. Chem. 278 (2003) 4145] apoptotic death of Leber's Hereditary Optic Neuropathy (LHON) cybrids (11778, 3460, and 14484 mutations) in galactose medium. To better define the apoptotic pathway, we assayed ATP synthesis driven by complex I/II substrates in permeabilized cells, as well as the cellular ATP content. All evaluations were corrected for citrate synthase activity. The ATP content was also assayed during a time-course experiment, after galactose replacement, as well as the activation of caspase 3 and the release of Endonuclease G (EndoG). Complex I-dependent ATP synthesis was severely affected with all three mutations. However, total ATP content of LHON cybrids was only slightly reduced compared to controls. In galactose medium, LHON cybrids suffered a profound ATP depletion with time. Despite the significant release of cytochrome *c* from mitochondria, caspase-3 was not activated. Mitochondrial EndoG levels decreased in LHON cybrids after 16–48 h of galactose incubation while cytosolic Endo G concurrently increased. This was not observed in control cells. Our results reveal that LHON mutations profoundly impair complex I-dependent synthesis of ATP. However, compensatory mechanisms maintain an almost normal content of total cellular ATP. LHON cybrid apoptotic death was caspase-independent, which may depend on the rapid ATP depletion suffered during galactose incubation. The parallel release of EndoG from mitochondria into the cytosolic fraction was probably responsible for nuclear DNA laddering and apoptotic death. Bioenergetic failure may therefore be more relevant than previously believed in shaping retinal ganglion cell death in LHON. Supported by Telethon-Italy (grant #GGP02323 to V.C.).

P-127
Mutations in AAC2, equivalent to human adPEO-associated ANT1 mutations, lead to defective oxidative phosphorylation in *Saccharomyces cerevisiae* and affect mitochondrial DNA stability

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Autosomal dominant external progressive ophthalmoplegia (adPEO) is a mitochondrial disorder associated with the presence of multiple deletions of mtDNA. Four adPEO-associated missense mutations have been identified in the ANT1 gene encoding the muscle-heart specific isoform of the mitochondrial adenine nucleotide translocator. Since ANT1 is not expressed in cultured fibroblasts or myoblasts, to investigate their consequences on cellular physiology, we introduced adPEO-associated ANT1 mutations at equivalent positions in AAC2, the yeast orthologue of human ANT1. Their expression in aac2-defective haploid strains of *Saccharomyces cerevisiae* resulted in a marked growth defect on non-fermentable carbon sources, and a concurrent reduction of the amount of mitochondrial cytochromes, cytochrome *c* oxidase activity and cellular respiration. Upon solubilization from mitochondrial membranes and functional reconstitution into liposomes, the AAC2 pathogenic mutants showed a marked ATP>ADP preference compared to wild-type AAC2. Insertion of the aac2 mutant alleles in combination with the endogenous wild-type AAC2 gene caused a significant reduction in cytochrome content and increased mtDNA instability. The

analysis of petite mutants in the heteroallelic strains AAC2/aac2A128P and AAC2/aac2S303M showed that all of them were rho − , indicating that neither the synthesis of mtDNA nor its segregation was inhibited. The increase in the rate of petite colonies observed in our mutant strains can account for the slow, progressive accumulation of multiple deletions in human mtDNA which is proposed to lead to adPEO. These results validate the yeast *S. cerevisiae* as a suitable in vivo model to further study the early biochemical and cellular consequences of adPEO-associated ANT1 mutations.

P-128
Tissue-specific cytochrome *c* oxidase assembly defects due to mutation in SCO2

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Sco2 protein involved in cytochrome *c* oxidase (COX) assembly presumably affects provision of Cu+ to CuA site of MTCO2. We studied the interactions and levels of COX subunits in heart, skeletal muscle and liver from three patients with COX deficiency and mutations in SCO2 (two G1541A homozygotes and one C1280T/G1541A compound heterozygote). *Methods*: Activity of COX was determined spectrophotometricaly. Interactions and levels of COX subunits were characterised by 1D-PAGE (native), 2D-PAGE (native/denaturing), SDS-PAGE and immunoblot analysis. *Results*: The activity of COX was markedly decreased in heart and skeletal muscle but only a mild decrease was found in liver and cultured fibroblasts. Tissue-specific COX assembly defects were found with decreased levels of holo-enzyme complex and increased levels of specific assembly intermediates. In the liver, most of the relevant cross-reactive material was located in holo-enzyme spots whereas in heart and skeletal muscle were detected severely decreased levels of holo-enzyme and markedly increased levels of assembly intermediates. Heart and skeletal muscle contained three major faster migrating assembly intermediates: the first one comprised only MTCO1, the second MTCO1, COX4 and COX5A, and the third contained at least MTCO1, COX4, COX5A and MTCO2. The tissues from C1280T/G1541A heterozygote showed apparently more severe assembly defects and lower steady-state levels of COX subunits than tissues from G1541A homozygotes. *Conclusions*: We demonstrate that tissue-specific decrease in COX activity in patients with SCO2 mutations is associated with similar extent of tissue-specific COX assembly impairment, resulting in decreased levels of holo-enzyme complex and increased levels of assembly intermediates. Supported by GACR-303/03/H065.

P-129
Intracellular ATP depletion and increase of oxidized glutathione in cultured cystinotic fibroblasts

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Background: Cystinosis is characterized by a defective transport of amino acid cystine out of lysosomes due to mutations of CTNS gene, encoding lysosomal cystine carrier. Based on in vitro studies of proximal tubular cells loaded with cystine dimethyl esters, ATP depletion is supposed to cause renal Fanconi syndrome in cystinosis. Increased excretion of 5-oxoproline in patients not treated with cysteamine raises the hypothesis of the alteration in gamma-glutamyl cycle.

The aim of the study was to investigate whether intracellular ATP and glutathione content were depleted in cultured fibroblasts of patients with cystinosis.

Methods: Intracellular cystine, total and oxidized glutathione and ATP content as well as activity of Na-K ATPase and of mitochondrial respiratory chain complexes (I–IV) were measured in cultured fibroblasts of eight cystinotic patients and eight healthy controls. Mann–Whitney test was used for statistical analysis.

Results: Median fibroblast cystine content of patients with cystinosis was 9.3 compared to 0.4 in healthy controls ($P < 0.01$). Total glutathione content did not differ between cystinotic and control fibroblasts, while oxidized glutathione was significantly higher in the patients (median 0.9 versus 0.23 nmol/mg protein, $P < 0.05$). Median ATP content was lower in cystinotic fibroblasts compared to the controls (37.3 versus 51.5 nmol/mg protein, $P < 0.05$). No difference in the activity of Na-K ATPase and mitochondrial respiratory chain complexes was detected.

Conclusion: ATP depletion found in cystinotic fibroblasts did not result in decreased activity of Na-K ATPase.

The elevation of oxidized gluthatione points to increased oxidative stress, which can be responsible for cell damage in cystinosis.

P-130
Skeletal muscle gene expression profiling in mitochondrial disorders

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Extremely variable clinic and genetic features characterize Mitochondrial Disorders (MD). Pathogenic mito-

chondrial genome defects are large-scale rearrangements and single point mutations. Clinical manifestations become evident when a threshold percentage of DNA molecules are mutated. The severity of the clinical phenotypes can be directly related to the heteroplasmic mutant load; however, other factors may contribute to the wide range of phenotypes associated with the same mutation. These complex genotype–phenotype relations must be governed by nucleo-mitochondrial interactions, but the precise nature of this genomic ''cross-talk'' is unknown. Using Affymetrix microarrays (HG-U133A), we studied the gene expression profile of muscle biopsies obtained from four patients carrying the ''common'' macrodeletion and eight A3243G (four PEO and four MELAS phenotypes) compared with three age-matched healthy individuals. We found several differentially expressed genes: 33 were markedly up-regulated in macrodeleted and four decreased; 57 genes were dysregulated in A3243G-related disorders (53 down-regulated in PEO and four up-regulated in MELAS). Eleven genes were similarly differentially expressed in MD muscle tissues when compared to healthy muscle samples. An increased expression of genes mainly related to the metabolism of the amino groups (most of them implicated in the Urea-cycle), as well as of several genes involved in the genetic information processing, contributed to this differential profile. Moreover, few genes were similarly decreased in MD patients versus the control group. Real-time PCR demonstrated excellent reproducibility of the microarray-based findings. The observed expression patterns may reflect specific changes of nuclear genes and contribute to the definition of a molecular signature for the MD.

P-131

Analysis of the ANT1 gene in progressive external ophthalmoplegia with multiple mtDNA deletions: identification of a novel mutation

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Progressive external ophthalmoplegia (PEO) associated with multiple deletions of mitochondrial DNA (mtDNA) can be caused by adenine nucleotide translocator 1 (ANT1) mutations. Up to now, only four mutations have been identified in the ANT1 gene in only a handful of PEO patients. We screened 29 index patients with PEO (16 sporadic and 13 familial cases) with multiple mtDNA deletions for ANT1 mutations. In one German family we identified a novel heterozygous C-to-A transversion at nucleotide 269. The mutation converted a highly conserved alanine at codon 90 to aspartic acid (A90D) and was identified in three siblings: two brothers presented with PEO and limb muscle involvement and the sister suffered from ptosis and schizoaffective disorder. Microsatellite analysis was suggestive of autosomal dominant inheritance from the mother who did not carry the mutation in blood indicating germline mosaicism. In 28 patients no mutation was found in the ANT1 gene. This study expands the heterogeneity of ANT1 mutations and confirms that multisystemic involvement seems to be not very frequent in PEO patients with ANT1 mutations apart from psychiatric symptoms. Furthermore, it underlines that ANT1 mutations are very rare in patients with PEO and multiple mtDNA deletions.

P-132

The 7472insC deafness-associated mitochondrial DNA mutation impairs 5′ and 3′ processing of pre-tRNA Ser(UCN)

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The 7472insC mitochondrial DNA mutation, which templates the insertion of an extra G within the T-arm of tRNA Ser(UCN), is associated mainly with sensorineural deafness. In cultured cybrid cells containing 100% mutant mtDNA, it causes a 65% decrease in the steady-state level of tRNA Ser(UCN), which we showed previously was due to a posttranscriptional reduction in its rate of synthesis. In an attempt to define the precise pathogenic molecular mechanism of the 7472insC mutation, we have now analysed its effects on both 5′ and 3′ processing of pre-tRNA Ser(UCN) in vivo and in vitro.

The analysis of tRNA Ser(UCN) isolated from cybrid cells revealed a high frequency of misprocessing of tRNA carrying the mutation. Over 11% of molecules showed evidence of misprocessing, and many molecules that were 5′ misprocessed were also 3′ misprocessed. Such species were almost undetectable ($<1\%$) in control cells. In vitro assays using HeLa cell RNase P and mitochondrial tRNA 3′ processing endonuclease revealed that the efficiency of both 5′ and 3′ processing was impaired by the mutation. The effect on 3′ processing of the mutant substrate was dependent on whether it retained a 5′ leader. Furthermore, we observed 5′ misprocessing in vitro of artificially 3′ misprocessed substrates. We conclude that the mutation impairs tRNA Ser(UCN) synthesis by affecting several RNA processing steps, representing a novel pathological mechanism.

P-133

Proteomic techniques applied on skeletal muscle and fibroblasts in the patients with oxphos deficiencies

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Finding the underlying molecular defect in the patients with deficient activity in one of the OXPHOS complexes is not an easy task. Complementation studies can be used to decide whether mutation analysis should be performed in mtDNA or in nuDNA. These studies, however, can be laborious. A diagnostic strategy was developed based on the study of the abundance of the complexes and their subunits for prediction of the location of the primary defect. Immunohistochemical and immunocytochemical analysis was performed in skeletal muscle and cultured skin fibroblasts using specific antibodies against OXPHOS subunits. In isolated mitochondria, OXPHOS complexes were separated by blue native polyacrylamide gel electrophoresis (BN-PAGE) and estimation of the abundance of each complex was performed using immunoblotting techniques. Two-dimensional electrophoresis (BN-PAGE/SDS-PAGE) was applied for separation of the subunits. Spots on the 2D gels were identified by immunostaining and mass spectrometric sequence analysis. mtDNA mutations affect the intramitochondrial protein synthesis. These mutations result in a reduced immunoreactivity in BN-PAGE gels for at least two complexes (I and IV mostly), and in a mosaic pattern on immunostaining of tissues and cells (using antibodies against complexes I and IV). Mutations in the nuclearly located assembly genes like SURF-1, BCS1L and ATP12 and in the nuclear genes coding for structural proteins were associated with a reduced immunoreactivity of only one complex, which was generalized. The combination of these proteomic techniques can diminish the number of relevant genetic tests and facilitate the identification of the underlying gene defect.

P-134

Is the mtDNA mutation A8348G pathogenic in patient with hypertrophic cardiomyopathy?

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The association of hypertrophic cardiomyopathy and heteroplasmic mtDNA mutation A8348G in TΨC loop of tRNALys has been described only once (Terasaki et al., 2001). We present second three-generational family with mtDNA mutation A8348G. The proband with exercise intolerance and progressive hypertrophic cardiomyopathy died at the age of 22 years due to heart failure with ventricular dilatation. *Results*: The heart biopsy revealed irregular decrease of COX and NADH diphorase activities with increased SDH activity. In isolated muscle mitochondria from muscle biopsy, spectrophotometric analyses revealed decreased activity of complex I (40% of controls), complex I + III (30% of controls), and complex IV (20% of controls) and 2D-PAGE has shown decreased amount of low-weight subunits of complex IV. On the contrary, no substantial alterations were found in muscle and skin biopsy using histochemistry and ELMI. At autopsy, excentric heart hypertrophy (980 g, controls < 300 g) was found with increased amount of mitochondria in some cardiocytes. Distribution of mitochondria in the liver and kidney followed by immunohistochemistry revealed only moderate variation of their number. The brain was edematous, of normal weight and without overt macroscopic abnormalities. Western blot analysis of 2D-PAGE revealed abnormal pattern of COX assembly in heart. *Conclusions*: We speculate that mtDNA mutation A8348G may hinder protein synthesis in mitochondria, which may affect assembly/stability and activity of complex IV in the heart. Because the other seven family members with mtDNA mutation A8348G are asymptotic, another molecular background for severe heart impairment in our patient cannot be excluded. Supported by IGA NR/8065-3 and GAÈR 303/03/D132.

P-135

The mitochondrial prohibitin complex is essential for embryonic viability, germline function and mitochondrial morphology in *Caenorhabditis elegans*

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Prohibitins in eukaryotes consist of two subunits (PHB1 and PHB2) that together form a high molecular weight complex in the mitochondrial inner membrane. The evolutionary conservation and the ubiquitous expression in mammalian tissues of the prohibitin complex suggest an important function among eukaryotes. The PHB complex has been shown to play a role in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes in the yeast *Saccharomyces cerevisiae*. We have used *Caenorhabditis elegans* as model system to study the role of the PHB complex during development of a multicellular organism. We demonstrate that prohibitins in *C. elegans*

form a high molecular weight complex in the mitochondrial inner membrane similar to that of yeast and humans. By using RNA-mediated gene inactivation, we show that PHB proteins are essential during embryonic development and are required for somatic and germline differentiation in the larval gonad. We further demonstrate that a deficiency in PHB proteins results in altered mitochondrial biogenesis in body wall muscle cells. We report a strong loss of function phenotype for prohibitin gene inactivation in a multicellular organism and show for the first time that prohibitins serve an essential role in mitochondrial function during organismal development.

P-136
The mitochondrial RNA polymerase is directly responsible for promoter recognition in mammalian cells

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Characterisation of the basic mammalian mitochondrial DNA (mtDNA) transcription machinery is of fundamental biological interest and may also provide novel pathways for therapeutic intervention in the large group of human diseases associated with mitochondrial dysfunction. We have previously identified two novel transcription factors, transcription factors B1 (TFB1M) and B2 (TFB2M), and demonstrated that they can each support promoter-specific mtDNA transcription in a pure recombinant in vitro system containing mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM, previously mtTFA). We have now investigated how the mitochondrial transcription machinery recognizes promoters and initiates RNA synthesis. Site-specific mutagenesis of the mitochondrial light-strand promoter allows us to define essential elements required for transcription. We furthermore demonstrate that recombinant in vitro transcription systems reconstituted from mouse and human cells only can recognize the species-specific and not the heterologous light-strand promoter. By swapping transcription factors between the mouse and human transcription machineries, we demonstrate that the observed promoter specificity is governed by the mitochondrial RNA polymerase and the mitochondrial transcription factor A. In contrast, transcription factor B does not influence promoter recognition at all. The RNA polymerse specifically recognizes sequence elements localized between nt -1 and -14 relative to the transcription start site, much similar to what has previously been described for the homologous RNA polymerase encoded by bacteriophage T7. Our studies lead us to propose a new model on how mitochondrial promoters are recognized and transcription initiated in the mammalian mitochondrion.

P-137
Spatial dynamics of human mitochondrial nucleoids during mitosis and apoptosis

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Here we describe a simple and effective method for the in situ visualisation of human mitochondrial DNA (mtDNA) using the DNA specific dye picogreen. By this method mtDNA is visualised as punctate, brightly fluorescent structures distributed throughout the mitochondrial reticulum in a variety of cell types. The staining is superior to other DNA dyes such as DAPI and ethidium bromide and can be viewed using a basic eppifluorescent microscope equipped with standard filters. The method is also well suited to confocal microscopy and the superior fluorescence and localisation allows mtDNA nucleoids to be visualised and tracked over periods of several hours. We have applied this approach to studying nucleoid dynamics during normal mitochondrial fission and fusion and in cells undergoing mitosis and apoptosis revealing novel distribution dynamics. The method can also be used in conjunction with immunocytochemistry which has allowed us to study how human mtDNA interacts with the cytoskeletal components actin and vimentin and with mitochondrial transcription factor A.

P-138
Investigation of association between mitochondrial backgrounds of Leber's Hereditary Optic Neuropathy and Multiple Sclerosis in Iranian patients

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The hypothesis that mitochondrial DNA (mtDNA) may be implicated in susceptibility to Multiple Sclerosis (MS) is supported by an increasing number of reports. We studied the mitochondrial possible point mutations background for both diseases. In 56 Iranian MS patients we could not find any primary Leber's Hereditary Optic Neuropathy (LHON) point mutations. We also investigated the same mitochondrial haplogroup (J, K) in Iranian patients.

We studied 70 MS patients and 149 healthy controls for Haplogroup J and K with PCR RFLP method. Our result showed that 14 out of 70 are positive (20%) in MS group

and 14/149 (9.4%) in control group for J ($P < 0.05$); and 9/70 (13%) in MS group and 5 out of 70 (7%) in control group are positive for Haplogroup K ($P = 0.4$). Association between haplogroup J and Optic Neuritis was shown (significant at $P < 0.005$).

Our haplogroup investigation (J, M, BM and N) in LHON patients normal control group (149 controls for haplogroup J. and 246 controls for haplogroups M, BM and N, from different regions of Iran) showed relation between LHON and the haplogroups J, M and N. However, the results showed a slight relatedness between haplogroup BM and LHON.

Conclusion:

(1) LHON and MS had not the same haplogroups background as well as point mutations.
(2) This study showed that haplogroups J may be a risk factor for MS diseases special for MS patient with Optic neuritis but genetic susceptibility factors for these disorders vary between different populations.
(3) There is a slight relatedness between haplogroup BM and LHON.

P-139
Progressive muscle atrophy with selective loss of muscle fibers in a patient with mtDNA TK2 deficiency

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Mutations in thymidine kinase 2 (TK2) gene cause a myopathic form of the mitochondrial DNA depletion syndrome (MDS). Although clinical manifestations in these patients vary, most of them die during infancy and long-term survival is unusual.

Here, we report the morphological and molecular findings, as well as a 12-year clinical follow-up in a patient with the myopathic form of MDS in whom pathogenic mutations were identified in the TK2 gene.

Morphological studies were performed in muscle biopsies that were obtained at ages 2, 5, and 12. Quadriceps muscle biopsy performed at age 2 depicted an extensive muscle fiber necrosis and phagocytosis, moderate fibrosis and numerous scattered fibers with diffuse increased oxidative activity and neutral lipid accumulation. At age 5, biopsy showed that the OXPHOS system was mildly affected but completely normal at age 12. At this age, biopsy showed a fairly well structured muscle integrated almost exclusively by type I fibers. Other findings include minimal lipid vacuoles and the presence of few, scattered COX negative ragged red fibers. mtDNA depletion worsened over the time (from 75% at age 5 to 90% at age 12). TK2 activity in fibroblasts was significantly reduced (7.8% of controls) and molecular analysis revealed the presence of two allelic heterozygous mutations in the TK2 gene (T108M in exon 5 and R192K in exon 8).

We propose that mtDNA-depleted fibers undergo selective segmental necrosis and, with time, they progressively vanish leading to muscle atrophy and weakness.

P-140
Hunting for a gene responsible for coenzyme Q10 deficiency

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Coenzyme Q10 (CoQ10) is a component of the electron transport chain. It also plays a role as antioxidant and membrane stabilizer.

Primary CoQ10 deficiency is an apparently autosomal recessive disorder with heterogeneous clinical presentations: (1) a rare myopathic form, dominated by exercise intolerance, recurrent myoglobinuria and central nervous system dysfunction; (2) an infantile-onset encephalomyopathy with ataxia and renal disease; (3) Leigh syndrome.

In 2001, we described a syndrome with cerebellar ataxia and severe deficiency of CoQ10 in skeletal muscle. Known genetic causes of cerebellar ataxia had been excluded; we suggested that this was a new form of primary CoQ10 deficiency. To identify the genetic cause of this disease we studied a family affected and we mapped the locus to chr. 9p13. We analyzed 10 genes as potential candidates and in three index patients of the family we found a nonsense homozygous mutation in a gene encoding a protein predicted to be targeted to mitochondria. We posit that a form of CoQ10 deficiency may be caused by mutations in this gene leading to mitochondrial respiratory chain impairments. In addition, low levels of CoQ10 may lead to oxidative stress.

P-141
Cell culture models for mitochondrial neurogastrointestinal encephalomyopathy

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder caused by

loss-of-function mutations in the gene encoding thymidine phosphorylase (TP). Muscle biopsies of patients have revealed morphological and biochemical abnormalities of mitochondria as well as multiple deletions, depletion and point mutations of mitochondrial DNA (mtDNA).

Normally, TP breaks down thymidine to thymine and ribose. In MNGIE patients, TP activity is very low or absent resulting in elevated levels of plasma thymidine. To explain how TP gene mutations cause mtDNA defects, we have hypothesized that elevated levels of thymidine cause mitochondrial nucleotide pool imbalances, resulting in abnormalities of mtDNA.

We are attempting to generate in vitro mtDNA abnormalities by exposing cultured TP-deficient and control cells to varying concentrations of thymidine. We transformed control and patient fibroblasts with SV40 and treated these immortalized cells with ethidium bromide to deplete mtDNA completely. These rho0 cells will be repopulated with wild-type mtDNA by fusion with normal cytoplasts generating cybrids with nuclei from either patients or healthy controls.

We will assess whether the cells accumulate higher heteroplasmic levels of mtDNA mutations over time, leading to significant mitochondrial respiratory chain defect, as assessed by histochemical stains and measuring biochemical activities for COX and SDH. We will screen mtDNA for point mutations by RFLP and for depletion and multiple deletions by Southern blot.

If the accumulation of mtDNA abnormalities in MNGIE fibroblasts is due to elevated thymidine levels in the culture medium, this result will encourage us to attempt therapies to eliminate circulating thymidine in MNGIE patients.

P-142
Hypertrichosis and SURF1 mutations

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We present two patients with generalised hypertrichosis and Leigh syndrome with COX defects due to mutations in SURF1.

Hypertrichosis may be seen in association with a number of diseases. The basic abnormality is often a conversion of vellus hair to terminal hair, a process similar to the normal response of the axillae and groin hairs to the increased androgen production in adolescence. The other mechanism of hypertrichosis is a change of the hair-growth cycle, caused by, e.g. increased thyroid hormone, growth hormone or androgens.

Hypertrichosis has previously been reported in three patients with mutations in SURF1. In a few patients with MELAS syndrome, hypertrichosis has been reported as well, but the mechanism behind the hypertrichosis in respiratory chain disorders is not known and most patients do not have hypertrichosis.

The two patients, a boy and a girl, both had noncongenital hypertrichosis. The girl, who is 3 years old, had highly elevated testosterone levels in blood: 4.96 mmol/l (ref. 1.1 ± 0.7).

The elevated testosterone level is likely the cause of the hypertrichosis, and may be caused by either an unknown effect of the SURF1 mutations on hormone metabolism, or by the bad condition of the patient, something that may be seen also in, e.g. anorexia nervosa.

In conclusion, hypertrichosis may be a frequent finding in patients with SURF1 mutations and in some cases probably caused by increased androgen levels.

P-143
Mitochondrial DNA sequence variation in patients with CADASIL

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Mutations in Notch3 gene cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) that is clinically characterized by recurrent ischemic strokes, migraine with aura, psychiatric symptoms, cognitive decline and dementia. We recently described a patient with CADASIL caused by R133C mutation in Notch3 and with concomitant myopathy caused by a novel 5650G>A mutation in the tRNAAla gene in mitochondrial DNA (mtDNA). We assume that the co-occurrence of the two mutations is not coincidental and that mutations in the Notch3 gene may predispose mtDNA to mutations. We examined the nucleotide variation in mtDNA coding region sequences of 87 individuals with CADASIL caused by mutations in the Notch3 gene. The patients were from 21 nuclear families. Conformation sensitive gel electrophoresis and direct sequencing were used to detect mutations and polymorphisms. Novel sequence variants were verified by restriction fragment analysis or allele-specific PCR. MtDNA sequence variation among the CADASIL patients was compared to that among 192 healthy Finns. We found a total of 197 mtDNA coding region sequence variants among patients with CADASIL. The variants included 10 novel synonymous, six novel nonsynonymous, two novel tRNA and two novel rRNA mutations. The variation was larger than expected. Our results suggest that mtDNA sequence variation is increased in patients with CADASIL

and point to the possibility of a relationship between Notch3 and mtDNA.

P-144
Leigh Syndrome French Canadian Cytochrome Oxidase deficiency due to an inherited mutation in the LRPPRC gene produces a defect in translation of COX subunits

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Leigh Syndrome French Canadian (LSFC) is a variant of cytochrome oxidase (COX) deficiency caused by mutations in the LRPPRC gene. It is an unusual form of COX deficiency with almost normal expression of COX in heart, skeletal muscle and kidney but with distinct deficiency in brain liver and skin fibroblasts. In patients with the common (A354V) founder mutation, the amount of LRPPRC protein found in both fibroblast and liver mitochondria was consistently reduced to $<25\%$ of control levels. Immunofluorescence showed that LRPPRC had a mitochondrial distribution in normal cells but was more diffusely distributed in the fibroblasts of mutant cells. A titre of LRPPRC was also found in nuclear fractions. Northern blots showed that the LRPPRC protein was expressed in skeletal muscle>heart>placenta>kidney>liver>lung = brain, opposite to the severity of the enzymatic cytochrome oxidase defect. 35S-Methionine labeling of mitochondrial translation products showed that the translations of COX I and COX III were specifically affected. This was also seen in Northern blots where LSFC cells had lower titres of COX I and COX III transcripts relative to those of ND1 and actin. These results suggest that LRPPRC has a role in the stability of the mRNA for COX I and COX III.

P-145
Apoptosis in muscle with mitochondrial DNA deletion

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Mitochondrial DNA (mtDNA) deletions were the first mtDNA alterations identified as the cause of mitochondrial diseases. They have been associated with three clinical presentations: two severe multisystemic diseases with early onset (Pearson and Kearns Shy Sayre (KSS) syndromes) and a milder adult disease with only muscle involvement (Chronic Progressive External Ophthalmoplegia, CPEO). However, many adult cases present with a multisystemic disease but an evolution much less severe than typical KSS (CPEO+). Molecular mechanisms underlying the difference of severity encompass tissue distribution, proportion, and location of mtDNA deletion as well as the mechanisms associated with the defect of the respiratory chain activities such as apoptosis and overproduction of reactive oxygen species (ROS). We have analysed the muscle biopsy of 16 patients with mtDNA deletion and diverse clinical presentation. Serial sections were investigated for the presence of defect of respiratory chain activity (COX histochemistry), mitochondrial proliferation (SDH histochemistry), apoptosis (caspase 3, Bax, TUNEL) and overproduction of ROS (SOD). The study of more than 2000 muscle fibres showed that apoptosis and ROS production are associated with the respiratory chain defect and mitochondrial proliferation.

To correlate these parameters with their genetic cause, the borders of the deletions were identified and the amounts of deleted and wild-type mtDNA were evaluated using real time PCR. The proportion of muscle fibres with diverse alterations was correlated with the amount of both deleted and wild-type mtDNA molecules in the muscle fragment. This same correlation will be performed at the level of individual muscle fibres.

P-146
Bioenergetic consequences of homoplasmic LHON mutations

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In the present report we investigated the bioenergetic consequences of 3 different homoplasmic LHON (Leber's hereditary optic neuropathy) mutations affecting the mitochondrial-encoded subunits of NADH:CoQ oxidoreductase ND2 (C4640A), ND4 (G11778A) and ND6 (T14484C) for human skeletal muscle and brain (for the ND2 mutation only). All mutations caused in patients and non-affected carriers of the mutation an almost identical decrease of citrate synthase-normalized activities of complex I being most severe for the ND6, less severe for ND4 and close to the detection limit for the ND2 mutation. This enzyme activity change was observed to be responsible for decreased respiration rates with NAD-dependent substrates detected in saponin-permeabilized muscle fibers, isolated skeletal muscle mitochondria and digitonin-treated parahippocampal homogenates (for the ND2 mutation). Titrations of the activity of NADH:CoQ oxidoreductase with the complex I inhibitors amytal, rotenone and piericidin A revealed with all mutations no difference to controls, indicating no alteration of kinetic properties of the CoQ reduction site by any of the investigated mutations. Since all mutations led to a considerable increase of amytal sensitivity of mitochondrial respiration, our data are compatible

with the concept that the investigated mutations lead to a decreased quantity of the active NADH:CoQ oxidoreductase enzyme complex. This hypothesis was tested to be valid applying quantitative immunohistochemistry with antibodies against the nuclear-encoded 15-kDa subunit.

P-147
A family with pyruvate dehydrogenase complex deficiency due to a novel C>T substitution at nucleotide position 407 in exon 4 of the X-linked E1alpha gene

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The pyruvate dehydrogenase complex (PDHc) is a multienzyme complex which converts pyruvate to acetyl CoA. A deficiency of the PDHc leads to lactic acidemia. The major cause of PDHc deficiency is a defect in the E1alpha component of the complex. The gene is localised to Xp22.1. The aim of this study is to describe the molecular genetic findings, clinical and laboratory features in a family with PDHc deficiency and a novel mutation in the E1alpha gene. Onset of disease in the proband and his older brother was at 17 and 8 months, respectively. Both had a severe episode of muscle weakness and lactic acidosis. The mother had milder symptoms. Respiratory rates and enzyme activities were measured in isolated muscle mitochondria and showed a generally decreased activity with a decrease of pyruvate oxidation that was considerably more pronounced than that of glutamate. All patients had low lactate/pyruvate ratios consistent with PDH deficiency, which was confirmed by thrombocyte PDH measurements. Sequencing of the 11 exons of the PDH E1alpha gene was performed. The proband and his brother were found to be hemizygous for a 407C>T change in exon 4 of the gene. Their mother was found to be a carrier of the mutation. Due to the X-linked inheritance pattern combined with the overall results of clinical investigations, molecular genetic findings and the corresponding functional deficiency of the gene product, we believe that the A136V substitution in the PDHE1alpha gene is the mutation leading to PDHc deficiency in this family.

P-148
Mitosim—a tool for simulation of mitochondrial population dynamics

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We describe a software tool, mitosim, for simulation of mitochondrial population dynamics. Mitosim makes it possible to model division and mutations in mitochondrial DNA; destruction, division and fusion of mitochondria; and death and division of cells. The parameters controlling the simulations can be set in a separate parameter file and parameters are also state-dependent.

Mitosim is intended as a tool for the simulation of accumulation of mutations in mitochondrial DNA in various kinds of cell cultures, e.g. mitotically active cells cultivated in vitro with periodic passaging and postmitotic somatic cells in vivo. The tool can also be employed as a tool for trying out different hypotheses concerning the mechanisms of mitochondrial fusion and replication.

P-149
Splicing error in E1alpha pyruvate dehydrogenase (PDHA1) mRNA caused by a novel intronic mutation: implication of the SR protein SC35

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A boy with delayed development, lactic acidosis, displayed a decreased PDH activity in fibroblasts and lymphocytes, and protein analysis demonstrated a decrease of the E1a and E1b subunits of the PDH complex. Recently, an intronic point mutation was identified in his E1a PDH gene (PDHA1). In addition to the normal spliced mRNA, patient samples contained significant levels of an aberrantly spliced mRNA: this mRNA involved a switch to the use of a cryptic 5' donor splice site (intron 7 position 45), with retention of the corresponding intronic sequence. The DNA analysis found no mutation in the coding regions but revealed a hemizygous G-to-A substitution at position 26 of intron 7, between the normal exon 7 donor splice site and the cryptic donor splice site. Splicing experiments in COS-7 and HeLa cells demonstrated that this point mutation at intron 7 was responsible for the aberrant splicing. This mutation is unusual in that it generated a consensus binding motif for the splicing factor SC35, one of the best characterized SR proteins. In vitro splicing and UV cross-linking experiments demonstrated that the mutation generated a splicing enhancer motif specially recognized by SC35. Consistently, reducing SC35 protein levels in patient's fibroblasts, using small interfering RNAs, resulted in the almost complete disappearance of the aberrantly spliced E1a PDH mRNA. We here not only define a new mutation with its mechanism in human pathology, but our findings also illustrated more generally

that the SR proteins could be new promising targets for therapeutic agents.

P-150
The neuropathology of mitochondrial DNA disorders

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Diseases associated with mitochondrial DNA (mtDNA) mutations are increasingly recognised as an important cause of neurological disability. The involvement of the central nervous system (CNS) is prominent, progressive and may be the most disabling feature for patients. The neurological features include optic atrophy, deafness, seizures, dementia, ataxia, extrapyramidal features and stroke-like episodes. These features are extremely variable even between patients with the same mutation, and our understanding of the correlation between mtDNA defects and specific neurological features is at a rudimentary stage.

We have had the opportunity to correlate the clinical, neuroradiological and neuropathological features of several patients with mtDNA disease. In addition, we have evaluated enzyme activity in frozen brain sections to correlate biochemical changes with the level of individual mtDNA mutations in single neurons.

Our data shows that:

- There is a remarkable difference in the number of cytochrome *c* oxidase (COX)-deficient neurons in different regions of the brain.
- Pathological features, including neuronal loss, may be prominent in some CNS regions but this does not directly correlate with the percentage of surviving COX-deficient cells. This suggests either that loss of COX-deficient neurons has already occurred, or that COX deficiencies in other cell types contribute to the neuron loss.
- In single neurons, COX deficiency is associated with the highest level of mutated mtDNA.
- The ''threshold'' level, the level of mutation required to before a neuron becomes COX deficient, varies and in part may depend on the CNS region examined.

P-151
Mitochondrial DNA mutations in human colonic crypt stem cells—implications for function

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Colonic stem cells are pluripotent and give rise to all cell lineages within the crypt epithelium. These cells are not totally defined but it is generally accepted that a stem cell 'niche' consisting of several stem cells exists at the base of the colonic crypt. Recent studies have shown not only a very high incidence of mitochondrial DNA mutations in human colons, but also the presence of extensive respiratory chain deficiency within crypts. The high incidence of cytochrome *c* oxidase-deficient crypts raises important questions not only regarding the possible influence of the respiratory chain deficiency on stem cell and colonic crypt function, but also to their possible influence in the development of colorectal cancers. Here we present new data showing:

1. the true incidence of respiratory chain deficiency in normal human colonic epithelium as determined by both histochemical and immunohistochemical techniques;
2. immunohistochemical data indicating that the presence of respiratory chain deficiency may influence several aspects of colonic stem cell and epithelial cell function (cell proliferation, apoptosis and the APC pathway);
3. extensive sequencing studies of the mitochondrial genome which indicate that the respiratory chain deficiency may influence colonic crypt division creating clusters of crypts derived from the same stem cell.

We believe these observations are relevant to other human stem cell populations.

P-152
Rapid mutation screening of the mitochondrial genome

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Although complete sequencing of the mitochondrial genome (mtDNA) is well established in the research and diagnostic setting, it still remains relatively expensive, the analysis time-consuming, and there are concerns about missing low levels of mtDNA heteroplasmy (<30%). Denaturing high performance liquid chromatography (DHPLC) can be used to screen PCR-generated DNA fragments for mutations by the formation of heteroduplexes. We used the Transgenomic MitoScreen Assay Kit to screen the entire genome in 19 PCR fragments. These fragments were then digested and analysed on the Transgenomic WAVE at different melt temperatures. A total of 10 patients were included in the study with well-characterised mutations at different levels of heteroplasmy. The DHPLC method identified the

mutations within specific fragments for all of these individuals.

P-153
An adenine phosphoribosyltransferase-like protein is down-regulated in a patient with OXPHOS deficiency

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To investigate the molecular consequences of OXPHOS deficiencies in patients suffering from mitochondriopathies, we compared patient's proteomes to healthy individuals'. The mitochondrial proteome originated from primary cultured fibroblast cell lines. In one patient, a nuclear-encoded gene mutation of unknown origin was suspected since enzyme complex activity measurements proved dysfunction of succinate dehydrogenase (complex II) and ATPase (complex V) in skeletal muscle and liver, but no mutation in the mtDNA was found. The patient's proteome revealed a significant diminished expression of an unknown protein having 41.6% identity to the human adenine phosphoribosyltransferase (APRT) protein. So far, we found human liver and leiomyosarcoma EST's matching the APRT-like sequence. The enzymatic function of the APRT itself takes place in the well-known salvage pathway restoring the nucleotide pool in the cytoplasm. AMP is generated from adenine and phosphoribosyl pyrophosphate (PRPP) and is used as a substrate for the ADP synthesis. ADP is subsequently imported into the mitochondria and used as a substrate for the OXPHOS system. To date, it still remains unclear whether there is a specific APRT-related mitochondrial protein producing AMP already in the mitochondria itself. Therefore, the APRT-like protein could be either down-regulated due to a lower requirement of ADP precursor in OXPHOS deficiencies or due to a gene mutation.

P-154
Marked mtDNA mutational load in isolated blood CD34+ cells from healthy subjects

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The accumulation of somatic alterations affecting the mitochondrial DNA (mtDNA) has been observed in aging and in pathological conditions. Recently, high levels of mtDNA sequence heterogeneity have been reported in CD34+ cells from adult bone marrow, and, also, mtDNA mutations have been described in colonic crypt stem cells, associated with a significant biochemical defect in their progeny. In the present study, we investigated the possibility that mtDNA mutations might accumulate also in isolated blood CD34+ cells. Peripheral blood was collected from five healthy subjects (range 23–79 years) and mononucleated fraction recovered through a density gradient. CD34+ cells were obtained by positive selection with magnetic cell separation and with fluorescence-activated cell sorter. The D-loop and the coding region spanning nucleotides 3200–3700 were amplified and analysed.

The cloning-sequencing strategy allowed to demonstrate that mtDNA mutated molecules (16.7–90%) and high mutational burdens ($0.34 \times$ kb–$4.8 \times$ kb) are present in all samples. CD34+ cells had always similar or increased frequencies of mutations, compared to those observed in whole blood-derived samples. All CD34+ cells showed a random distribution of the mutation pattern within the regions analysed; no recurrent nucleotides or short subregions showed increased mutational frequencies; both regions were characterised by the presence of individually rare point mutations.

Our data suggest that the accumulation of somatic mtDNA mutations occurs on a frequent basis in CD34+ peripheral blood mononucleated cells. As previously observed, it may be assumed that the presence of a relatively high mtDNA mutation load does not select the CD34+ bone marrow subpopulation from the circulating blood mononucleated cells.

P-155
Mutation analysis of the D-Loop and the 12S and 16S rRNA genes in prostate cancer

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Prostate cancer (PC) is the most commonly diagnosed cancer among men in Western countries. It has been suggested that mtDNA mutations could provide a very useful tool for the early detection of cancer. This is particularly interesting in the case of PC for which there is no effective diagnostic marker as yet.

We studied the presence of mtDNA mutations in 26 samples of prostatic tissue: 11 pairs (normal-tumour) from individuals with prostate cancer, three benign prostate hyperplasias, and one young control. For all samples, 4 kilobases of mtDNA were amplified and sequenced in eight different reactions. This region encompassed the D-loop, as well as the 12S and 16S ribosomal RNA genes.

A total of 55 sequence variants were identified, and distributed as follows: 45 in the D-loop (four not listed neither

in the Mitomap nor the Human Mitochondrial Genome databases); five in the 12S rRNA gene (one unlisted); and five in the 16S rRNA gene. Changes were detected in normal, tumour and hyperplastic prostates. The normal samples and their counterparts exhibited the same set of sequence variants. None of the mutations identified thus far seems to be specific for carcinogenic growth. These are the first results from a much more extensive study currently in progress that includes the analysis of a larger number of samples and the sequencing of the whole mtDNA molecule.

P-156
Is a novel nucleotide change in ND3 pathogenic?

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Transmitochondrial cybrid studies had previously been carried out on a cell line from a patient presenting with cardiomyopathy and complex I and IV deficiency. A wild-type nucleus (from A549 cells) did not correct the COX activity and so the mutation was assumed to be mitochondrial. Sequencing of the entire mitochondrial genome revealed a novel change (A10306G) in the ND3 gene. This results in an asparagine to serine substitution at amino acid position 83, which appears to be only moderately conserved across a selection of mammals and other organisms. The change was also found in the unaffected mother at lower mutant loads but was not present in 746 controls. An assay was designed using last-cycle fluorescent PCR-RFLP to determine the degree of heteroplasmy in each of the 12 transmitochondrial cybrid cell lines. Because the percentage of A10306G in the cybrid cell lines was found to vary considerably, we attempted to use these cybrid cell lines to find functional evidence of pathogenicity. However, high enzyme activities did not correlate with a decrease in the mutant load, so it is thought that this change is simply a heteroplasmic polymorphism. The real pathogenic change in this patient has not been discovered. In cases where the mutant load in transmitochondrial cybrids does vary widely, the authors suggest that these can be used as a means of obtaining functional evidence for (or against) pathogenicity.

P-157
Towards stable allotopic expression of the human mitochondrial ATPase 6 (subunit a) gene

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One of the mutations of mitochondrial DNA (mtDNA) was localised to the MTATP6 gene that encodes subunit 6 of ATP synthase (ATP6). The mutation, a thymine to guanine transversion at nucleotide position 8993 of human mtDNA (T8993G), converts a highly conserved leucine to an arginine at amino acid position 156. The mutation impairs ATP synthesis. Disease manifests when T8993G accounts for 80–90% of all mtDNA molecules, in the form of maternally inherited neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP).

The approach we have adopted to complement the ATP synthesis deficiency associated with the T8993G mutation is termed allotopic expression and has been established in yeast. It entails introduction of a wild-type MTATP6 (nA6) into the nucleus. The nA6 gene has been fused with selected mitochondrial targeting sequences (MTS) joined at the 5′ -end to ensure the protein product is imported by mitochondria.

A neuronal cell line carrying the T8993G mutation has been established to provide an improved cell model. In preliminary transfection experiments with the nA6 gene, cell survival is greater for cells carrying the T8993G mutation than control cells, yet all the cells tested failed to maintain expression of nA6. As the protein is highly hydrophobic and mitochondrial import is inefficient, longer MTS are being tested. Alternative expression systems and co-expression of molecular chaperones are also under investigation. These modifications aim to limit the toxic effects of nA6 expression and provide mitochondria with sufficient copies of functional ATP synthase to reverse the ATP synthesis deficiency.

P-158
The role of mitochondrial ND1 gene mutations in the aetiology of MELAS

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Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) is one of many clinical presentations associated with complex I deficiency. MELAS is genetically heterogeneous, with about 80% of patients harbouring the 3243A>G mutation in MTTL1. Other mutations in MTTL1, other transfer RNA genes and MTND complex I structural subunit genes, most notably MTND5, have also been reported in patients with a MELAS phenotype. We report three unrelated children with MELAS who expressed a specific deficiency of complex I activity in skeletal muscle and cultured fibroblasts, and lacked the 3243A>G mutation. Each harboured a different novel mutation in the MTND1 subunit gene of

complex I: 3696G>A (G131S), 3946G>A (E214K) and 3949T>C (Y215H). All affect highly conserved residues that are predicted to be in hydrophilic loops that face the matrix. Complex I activity was not restored on fusion of patient cells with a rho zero cell line, confirming pathogenicity. BN-PAGE showed markedly reduced levels of fully assembled complex I, suggesting these mutations affect complex I assembly or turnover, and confirming a crucial role for ND1 in complex I assembly. MTND1 mutations have been associated predominantly with Leber Hereditary Optic Neuropathy, but only the 3460G>A mutation has unambiguous evidence for pathogenicity. With three new pathogenic mutations, our findings suggest MTND1 may be a hotspot for mutations causing MELAS.

P-159

Mitochondrial DNA mutations distinctively alter nuclear gene expression

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The pathobiochemical pathways determining the wide variability in phenotypic expression of mitochondrial DNA (mtDNA) mutations are not well understood. Most pathogenic mtDNA mutations induce a general defect in mitochondrial respiration and affect ATP synthesis. Yet phenotypic expression of the different mtDNA mutations shows large variations that are difficult to reconcile with ATP depletion as sole pathogenic factor, implying that additional factors contribute to the phenotype. Here we use DNA microarrays to identify the changes in nuclear gene expression resulting from the presence of the A3243G diabetogenic mutation and from a depletion of mtDNA ($\rho 0$ cells). We find that cells respond to these states of mitochondrial dysfunction with both general and mutation-specific changes in nuclear gene expression. This observation indicates that cells can sense the nature of mtDNA dysfunction. As a common response in A3243G and $\rho 0$ cells, the mRNA levels of genes involved in ubiquitin-mediated protein degradation and in ribosomal protein synthesis are down-regulated, while mRNA levels for extracellular matrix genes are up-regulated. In contrast, a number of other genes show divergence in expression in $\rho 0$ cells compared to cells with the A3243G mutation, such as genes involved in oxidative phosphorylation. Our findings show that mitochondrial dysfunction by different mutations affects nuclear gene expression in partially distinct ways, suggesting that multiple pathways link mitochondrial function to nuclear gene expression and contribute to the development of the different phenotypes in mitochondrial disease.

P-160

Mutations of mtDNA in children with isolated complex I deficiency

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Complex I (NADH dehydrogenase) of the mitochondrial respiratory chain consists of seven mtDNA-encoded and ~ 39 nucleus-encoded subunits. Complex I has several biochemical activities including NADH oxidation, electron transport, proton pumping, coenzyme Q reduction and formation of reactive oxygen species. A variety of different clinical phenotypes are associated with complex I deficiency, but the pathogenesis is not well understood. We have diagnosed isolated complex I deficiency in 11 children by measurement of the mitochondrial ATP production rate (MAPR) and complex I enzyme activity in isolated skeletal muscle mitochondria. We divided the patients into four clinical groups based on their symptoms: (i) Leigh and Leigh-like syndrome ($n = 3$ patients); (ii) neonatal lactic acidosis, encephalomyopathy and hypertrophic cardiomyopathy ($n = 3$); (iii) encephalomyopathy with hearing impairment, optic nerve atrophy and cardiac involvement ($n = 4$; all siblings); (iv) hearing impairment, cataract, muscle weakness and hypertrichosis ($n = 1$). Five of the patients had elevated blood lactate, which in all cases was accompanied by excretion of lactate and Krebs cycle intermediates, in particular fumarate, in the urine.

Conformation sensitive gel electrophoresis (CSGE) was used to analyze 63 DNA fragments corresponding to the coding region of mtDNA in 10 of the patients. All fragments with aberrant migration were sequenced. Fragments with mtDNA mutations corresponding to known polymorphisms were not further investigated. Previously unreported polymorphisms/mutations or known pathogenic mutations were confirmed by sequencing in the reverse direction and by restriction fragment length

polymorphism analysis. Two patients with Leigh syndrome (group I) had the 10191T → C mutation in the ND3 gene and the 14487T → C mutation in the ND6 gene, respectively. The four patients of group III were siblings and they were all homoplasmic for the 11778G → A mutation of the ND4 gene. The 11778G → A mutation causes Leber's hereditary optic neuropathy (LHON) and may therefore explain the finding of optic atrophy in these patients. However, the patients also had encephalopathy and hearing impairment, which may represent a novel phenotype caused by the 11778G → A mutation. Alternately, the patients may also have a nuclear mutation explaining part of their phenotype. In conclusion, we demonstrate here that a variety of phenotypes may be present in children with complex I deficiency; our findings indicate that mutations of mtDNA may be common in children with Leigh syndrome and complex I deficiency.

P-161
Isolated Complex I deficiency results in increased superoxide production: a single-cell study

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Human Isolated Complex I deficiency is the most frequent encountered abnormality of the mitochondrial oxidative phosphorylation system. Complex I (CI) is a multi-subunit protein complex that dehydrogenates NADH and shuttles electrons to ubiquinone. Recently, the Nijmegen Centre for Mitochondrial Disorders of the department of Pediatrics has identified mutations in nuclear DNA, encoding several structural subunits of CI in patients with an isolated CI deficiency. To date, the mechanisms by which such mutations produce cell injury are poorly understood. One hypothesis is that mutations in CI subunits increase electron leakage which subsequently can react with oxygen resulting in an increased amount of damaging reactive oxygen species, especially superoxide. To test this hypothesis, we used the superoxide specific fluorescent probe dihydroethidium to measure cellular superoxide production at single cell level. Studies revealed that patient fibroblasts, in which a CI deficiency was determined, displayed significantly elevated superoxide production compared to controls. Furthermore, superoxide production from these cells inversely correlated with residual CI activity found by enzymatic analysis. This indicates that in patients more superoxide is produced by a smaller amount of active (though mutated) CI, compared to greater CI amounts in wild-type controls.

Further experiments will be conducted to gain more insight in the cellular superoxide detoxification mechanisms in patient and control fibroblasts and to restore the observed superoxide overproduction to control levels using a baculovirus-based complementation assay.

P-162
The absence of apoptosis-inducing factor AIF induces complex I deficiency

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Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein that, after apoptosis induction, translocates to the nucleus where it participates in apoptotic chromatinolysis. We show that mouse cells lacking AIF as a result of homologous recombination exhibit a high lactate production and an enhanced dependency on glycolytic ATP generation. This is due to a severe reduction of the respiratory chain complex I (CI) activity. We confirmed this CI deficiency in human cells in which AIF is knocked down by small interfering RNA. Although AIF does not co-migrate with CI, AIF-deficient cells lack selected CI subunits, pointing to a role of AIF in the biogenesis and/or maintenance of this polyprotein complex.

Harlequin mice, described by Klein et al. (Nature 2002), with reduced AIF expression due to a retroviral insertion into the AIF gene also manifest a reduced oxidative phosphorylation (OXPHOS) in the retina and in the brain, mostly CI, correlating with neuronal defects. Harlequin mice could therefore serve as an animal model for complex I deficiency.

Altogether, these data point to a role of AIF in the OXPHOS and emphasize the dual role of AIF in life and death.

P-163
Mmm1p spans both the outer and inner mitochondrial membranes and contains distinct domains for targeting and foci formation

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Maintenance of mitochondrial morphology is important for optimal function of the organelle. In *Saccharomyces cerevisiae*, the integral membrane protein Mmm1p is required for formation of tubular mitochondrial morphology and mitochondrial DNA (mtDNA) retention. Mmm1p forms discrete foci on the mitochondrial surface that are adjacent to mtDNA nucleoids in the matrix, raising the

possibility that this protein plays a direct role in anchoring mtDNA nucleoids. Mmm1p consists of a long N-terminal extension followed by a putative transmembrane segment and a large C-terminal domain. Although previous studies suggested that Mmm1p is inserted into the outer membrane with its C-terminus in the cytoplasm, the location of the N-terminus and the function of each domain remained unclear.

In this study, we examined the topology and function of different Mmm1p domains. We found that Mmm1p spans both the outer and inner mitochondrial membranes, with its C-terminus in the cytoplasm and its N-terminus in the matrix. Although this topology would allow the Mmm1p N-terminal extension to interact with mtDNA nucleoids, deletion of this region did not affect mitochondrial morphology or mtDNA retention. Moreover, the mtDNA loss phenotype of mmm1 null cells was rescued by expression of the *Neurospora crassa* Mmm1p homolog, which naturally lacks an N-terminal extension. These observations indicate that Mmm1p is not directly linked to mtDNA nucleoids. Additional studies showed that the transmembrane segment and C-terminal domain are essential for Mmm1p foci formation and mitochondrial targeting, respectively. Our data suggest that the double membrane-spanning topology of Mmm1p is crucial for maintenance of mitochondrial morphology.

P-164

Development of subdural effusions: pyruvate dehydrogenase complex (PDHc) deficiency can be mistaken for shaken baby syndrome

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A 3-month-old male infant was admitted in the pediatric department due to status epilepticus. Prior history was unremarkable with the exception of a UTI episode. A neonatal death was reported in the mother's family history. Physical examination revealed increased head circumference, retrognathia, dysmorphic ears, axial hypotonia and increased tendon reflexes. Fundoscopy demonstrated early signs of optic atrophy. Initial laboratory investigation was unrevealing. The MRI scan revealed a relative increase of extracerebral CSF spaces and generosity of the perimesencephalic cisterns. During the next 20 days, head circumference increased 10 cm. The subsequent MRI scan revealed subdural effusions (hygromas) and leukodystrophy. As the combination of macrocephaly, leukodystrophy, mild dysmorphias, and seizures is indicative of a mitochondrial disease, metabolic investigations were performed showing only ethylmalonic aciduria. A muscle biopsy was then performed revealing isolated PDHc deficiency (19 mU/U CS, normal range: 28–89 mU/U CS) with normal activities of E1 and E3 PDHc subunits.

The patient underwent surgical treatment of the hygromas and was placed under anticonvulsant therapy and ketogenic diet. A 15-month follow-up reveals severe psychomotor retardation, deviant developmental curve, brittle hair, axial hypotonia, limb spasticity and recurrent infections.

Spontaneous subdural effusions have been predominantly described in shaken baby syndrome, as a result of tear at the bridging veins during shaking of the infant: this was indeed the initial suspicion in this case. In the metabolic field, they are associated with glutaric aciduria type I. To our knowledge, this is the first described PDHc deficiency case associated with acquired subdural effusions.

P-165

Gene expression profiling of thyroid mitochondria rich tumours reveals a coordinated up-regulation of genes involved in oxidative metabolism

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Oncocytomas are a particular class of follicular thyroid neoplasms associated with a high cellular richness in functional mitochondria. In order to explore the mechanisms triggering this mitochondrial accumulation, we performed a gene expression profiling study of thyroid oncocytic tumours.

The mRNA from 29 thyroid oncocytomas and 58 samples including normal thyroid, thyroid papillary carcinomas, other thyroid pathologies, non thyroidal mitochondrial rich tumours and human thyroid cell lines were analysed using Nylon microarrays containing the PCR products for 6720 cDNA clones and radioactive labelling of the samples. Analysis of the data set produced was performed using supervised and unsupervised hierachical clustering.

These two approaches revealed two gene clusters specifically overexpressed in thyroid oncocytomas and 163 genes distinctly regulated between oncocytoma and normal thyroid. Differential expression for five genes (APOD, BCL-2, COX, CTSB and MAP2) was confirmed at the protein level using immunohistochemistry on 15 oncocytoma-normal thyroid couples. The two oncocytic specific clusters were rich in genes coding for mitochondrial proteins from both mitochondrial and nuclear genomes. The increase in mitochondrial biogenesis was confirmed by the overexpression

of genes involved in this process, such as NRF-1 or NOS3. Several genes involved in oxidative metabolic pathways (glycolysis, tricarboxylic acid cycle and oxidative phosphorylation) were up-regulated. Interestingly, the lactate dehydrogenase gene, involved in anaerobic metabolism, was down-regulated in oncocytomas.

Our gene expression profiling of thyroid oncocytoma revealed a coordinated overexpression of oxidative metabolism genes in these tumours. Our results suggest that, unlike a majority of solid tumours, thyroid oncocytoma produce energy through an aerobic pathway.

P-166
Molecular mechanisms and biochemical consequences of 9205delTA mutation in ATP6 gene

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We have investigated a new case of mtDNA mutation, 2-bp-deletion TA at position 9205, in ATP6 gene that was present at increasing load in a three-generation family (blood-grandmother 16%, mother 82%, patient 98%). In the affected boy with severe encephalopathy, a homoplasmic 9205delTA mutation was found also in fibroblasts and skeletal muscle. Patient fibroblasts showed normal aurovertin-sensitive ATPase hydrolytic activity, 70% decrease of ATP synthesis and 85% decrease of cytochrome *c* oxidase (COX) activity. ADP-stimulated respiration and ADP-induced decrease of mitochondrial membrane potential (state 4) were 50% reduced. Subunit a content was 10-fold decreased compared to other ATPase subunits and [35S]methionine labelling showed pronounced decrease in subunit a biosynthesis. The content of COX subunits 1, 4 and 6c was 30–60% decreased. NB and QT/RT/PCR analysis further demonstrated that the primary ATP6-COX3 transcript is cleaved to ATP6 and COX3 mRNAs two- to threefold less efficiently. Structural studies by Blue-Native and 2D-electrophoresis revealed altered pattern of COX assembly and instability of the ATPase complex, which dissociated into subcomplexes. The results indicate that 9205delTA mutation, which affects ATP6 gene STOP codon and the cleavage site between ATP6 and COX3 RNAs, prevents the synthesis of ATPase subunit a and causes the formation of incomplete ATPase complexes that are capable of ATP hydrolysis but not ATP synthesis. Mutation also affects COX biogenesis that is present in decreased amount. Interestingly, unchanged RNA processing and only some accumulation of F1-intermediate were found in the only other known case of 9205delTA with much milder clinical presentation.

P-167
Mitochondrial ATPase 6 gene mutation in two brothers with Leigh syndrome

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Leigh syndrome (LS) is a progressive neurodegenerative disorder with variety in inheritance mode consisting of autosomal recessive, autosomal dominant, X-linked or maternal trait. Leigh syndrome results from several defects of mitochondrial oxidative phosphorylation including pyruvate dehydrogenase complex, NADH-ubiquinone oxidoreductase, cytochrome *c* oxidase and ATP synthase.

We present two brothers with severe clinical symptoms of Leigh syndrome. The first boy was hypotonic from the neonatal period and showed failure to thrive, dissociated eyes movements and developmental delay. At the age of 5 months he rapidly deteriorated during intercurrent infection and died. Autopsy revealed gray and white matter spongiform degeneration with small vessel proliferation. The second boy was slightly hypotonic but during first three months his development seemed to be normal. At age of 4 months after intercurrent infection, failure to thrive, bizarre movements of eye globes and progressive weakness began to develop. He died at the age of 6 months. Biochemical analysis revealed elevated lactate in serum and cerebrospinal fluid. Muscle biopsy showed only mild lipid accumulation. Brain CT revealed bilateral hypodensities in basal ganglia.

Genetic study was performed on DNA isolated from dry blood spots. Mitochondrial DNA and nuclear SURF1 gene were examined to look for pathogenic mutations. PCR-SSCP analysis excluded presence of any SURF1 mutations. Direct sequencing of mtDNA showed presence of homoplasmic T-to-G transversion at nucleotide position 8993 in the MTATP6 gene. The mutation resulted in change of a highly conserved hydrophobic leucine-156 to a hydrophilic arginine in subunit 6 (ATPase 6) of mitochondrial ATP synthase.

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P-168
MitoScreenä Assay Kit, a novel approach for rapid mutation screening of the entire mitochondrial genome using multiplex DHPLC

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Mutations of the mitochondrial genome are the cause of various disorders. Many of them involve multiple organ systems and present prominent neurological and/or myopathic features. Development of diabetes type II is also frequently observed. Mitochondrial DNA mutations have further been associated with cancer and aging.

Denaturing High Performance Liquid Chromatography (DHPLC) in combination with the WAVE® System enables automated mutation detection via heteroduplex analysis. This technique is capable of providing high efficiencies when scanning for unknown mutations. A further advantage is the ability to detect small proportions of a mutant allele in a wild-type population. This high level of sensitivity, which is particularly relevant in revealing heteroplasmic mtDNA mutations, has explicitly been documented in the context of mtDNA mutation discovery(1).

We will show the development of a novel assay kit facilitating the rapid, reliable and highly sensitive discovery of mtDNA mutations using the WAVE® System. Human total DNA is used as template for amplification of the mitochondrial genome using 19 primer sets. The resulting overlapping fragments range in size from 300 to 1500 bp. Four of these fragments can be used for conventional DHPLC analysis. The remaining 15 fragments undergo a subsequent restriction digestion step to produce a collection of fragments that can be analyzed by multiplex DHPLC.

Results presented address the following aspects: purity of primers for best PCR results, PCR protocol using a proof reading polymerase (Optimase® polymerase), restriction conditions, influence of DNA template concentration.

P-169

Complex I deficiency a central mechanism for energetic depression at mitochondrial disorders, aging, sepsis, cancerogenesis and intoxications

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Due to the complexity of energy metabolism the relations between mutation load, enzyme activity and functional deficits are not linear and often uncertain. The aim of the present investigation was to detect complex I (CI) caused dysfunction at different pathological states and to estimate the flux control coefficients (Co) of CI in intact myoblasts at acute inhibition by haloperidol.

Scaling the pyruvate-dependent respiration on the succinate respiration measured in the same sample (SRPR), we obtained a parameter which is sensitive to CI-changes but independent of the amount of mitochondria. SRPR was found to be $120 \pm 18\%$ in normal human muscle and $123 \pm 5\%$ in human atrium but reduced in muscle from patients with mitochondrial cytopathies (CPEO $79 \pm 21\%$, MELAS $100 \pm \%$), in atrium of patients with fibrillation $100 \pm 2\%$, and in several tumors (21–64%). Similar changes we detected in aged FISHER rats and in endothelial smooth muscle cells after incubation with TNFα and endotoxin. All these changes correlated with reduced activities of complex I and I + III.

As a model for a defined decrease of complex I in living cells we investigated the effect of haloperidol on intact myoblasts. Haloperidol (50 μM) caused a 50% inhibition of CI, increased Co of CI in intact cells from 0.13 to 0.46 and caused energetic depression as detected by impaired Ca^{2+} signalling.

The large variety of hereditary and acute CI-related impairments indicates that complex I is one central target at mitochondrial diseases.

P-170

In situ genotyping mitochondrial DNA suggests physical clustering of pathogenic alleles as a factor influencing segregation of mitochondrial point mutations in mitotic cells

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The mode of mitochondrial genome segregation in mitosis is currently obscure, but of fundamental importance for understanding the mechanism by which pathogenic mutations are purged from the population, and how they may become enriched in tissues of affected individuals. We have applied an in situ genotyping approach to visualize and quantify the distribution of pathogenic mtDNA variants within and among cells from a heteroplasmic cybrid cell line. By using padlock probes and fluorescence microscopy, we show that the mutant variant can be readily distinguished from the normal variant. We observed a high variability in heteroplasmy level among cells, indicating a rapid bidirectional drift from the initial heteroplasmy level of the founding cell. Also intracellular clustering of the respective variants was observed, which may underlie mechanisms of rapid drift.

P-171

Another patient with the T14487C mutation in mtND6: severe movement disorder and Leigh-like syndrome

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Very recently, a new mtND6 mutation, T14487C, was described in four patients presenting with ataxia, dystonic movements, severe spasticity and optic atrophy in two of them. These symptoms were associated with Leigh-like lesions on MRI. Here we describe another patient carrying the same mutation. The leading symptoms of our patient are dystonia, dysarthria, tremor and ataxia, which are associated with spasticity and optic atrophy; however, the mental functions are nearly normal. On muscle biopsy no typical ragged red fibers were noted, however, subsarcolemmal accumulation of enlarged mitochondria was present. Biochemical measurement of respiratory chain enzymes showed decreased activity of complex I. Sequencing of mtDNA identified a T14487C mutation in ND6 with a high rate of heteroplasmy both in skeletal muscle and leukocytes. This mutation was not detectable in leukocytes of the mother. Our case confirms that T14487C causes a Leigh-like syndrome, where the most prominent features are dystonia and ataxia. Optic atrophy is also present reminding of LHON plus dystonia symptomatology caused by other mutations of the same ND6 gene. These findings suggest that mtND6 and particularly the T14487C mutation should be studied in patients with maternally inherited or spontaneous Leigh-like syndrome and dystonia.

P-172
Stimulation of mitochondrial activity induces transformation and a defective myogenic phenotype in human dermal fibroblasts

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Mitochondrial dysfunctions are frequently reported in tumor tissues, but their involvement in tumorigenesis remains unclear. In this study, we have investigated the influence of a mitochondrial stimulation induced by overexpression of triiodothyronine (T3) receptors on human dermal fibroblasts.

Cells were stably transfected with an expression vector encoding nuclear (c-ErbAa1) and mitochondrial (p43) T3 receptors. Two clones (MT1 and MT2) were selected. We found that only p43 was synthesized and specifically addressed to mitochondria, leading to an increase in organelle activity. In contrast to control cells, these clones displayed strong morphological changes, fused into multinucleated structures and expressed muscle specific genes (Myf 5, b-myosin, connectin, AchRa). Moreover, MT1 and MT2 cells displayed all features of transformation: (i) serum or anchorage-independent growth; (ii) loss of contact inhibition; (iii) tumorigenicity in vivo. This transformed phenotype was associated to a rise in c-Jun and c-Fos expression, and a strong inhibition of the expression of tumour suppressor genes sensitive to methylation (p53, p21, Rb). Lastly, DNA methyltransferase activity and ROS production were increased in correlation to mitochondrial activity.

Inhibition of mitochondrial activity resulted in a partial restoration of the normal phenotype, with the loss of ability to fuse, a strong decrease in muscle-specific gene expression, and an inhibition of the transformed phenotype. However, expression of tumour suppressor genes was not restored.

In conclusion, stimulation of mitochondrial activity in human dermal fibroblasts induces cell transformation through events involving ROS production and stimulation of DNA methylation.

P-173
Application of the yeast *Yarrowia lipolytica* as a model to analyse human pathogenic mutations

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We have used the obligate aerobic yeast *Yarrowia lipolytica* to reconstruct and analyse human pathogenic mutations in nuclear-coded subunits of mitochondrial complex I. Homozygosity for an exon 2 skipping mutation in the NDUFV2 (24 kDa) gene had been found to cause hypertrophic cardiomyopathy and encephalomyopathy. Although the mitochondrial targeting sequence was partially deleted, mitochondrial import was not completely abolished. After reconstruction in *Y. lipolytica*, isolated mutant enzyme was indistinguishable from parental complex I with regard to activity, inhibitor sensitivity and EPR signature. Size and isoelectric point were altered, indicating that the residual targeting sequence was retained on the mature 24 kDa protein. Homozygosity for substitution V122M in NDUFS7 (PSST) and heterozygosity for substitutions P79L and R102H in NDUFS8 (TYKY) had been found to cause Leigh syndrome. After reconstruction in *Y. lipolytica*, mitochondrial membranes from all three mutant strains exhibited similar complex I defects, with V_{max} being reduced by about 50%. In addition, changes in the K_M for *n*-decyl-ubiquinone and in the I50 values for hydrophobic complex I inhibitors were observed. Mutations in the NDUFS2 (49-kDa subunit) gene had been found to cause cardiomyopathy and encephalomyopathy. After reconstruction in *Y. lipolytica*, mitochondrial membranes from mutants R228Q and S413P showed no differences to wild type with regard to K_M, V_{max}, and temperature stability, whereas no assembly of complex I was found in mutant P229Q. These results reveal that functionally critical regions in

many cases are conserved between mammalian and fungal complex I.

P-174
Two novel mutations in the mitochondrially encoded ATPase 6 gene

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Human ATP synthase, complex V, contains a catalytic domain (F1) and a proton channel domain (F0). The F0 domain comprises nine subunits including the mitochondrial DNA (mtDNA) encoded ATPase 6 and 8. Pathogenic mutations in ATPase 6 leading to partial complex V deficiency have been described in several neurological diseases, e.g. Leigh syndrome (LS). Here we have analysed the ATPase 6 and 8 genes in two children with LS. Biochemical analysis showed a clear reduction of ATPase activity in muscle mitochondria of both patients. Mutational analysis of ATPase 6 and 8 revealed a maternal inherited heteroplasmic T → C substitution (T9185C) in the ATPase 6 gene of patient 1, resulting in a substitution of a leucine to proline (L220P). The proportion of the mutation in the patient's blood and muscle was >96%. It was 85% in a blood sample of his asymptomatic mother. Patient 2 exhibited a novel and nearly homoplasmic T9191C missense mutation in the ATPase 6 gene, which changed a highly conserved leucine to proline (L222P) of the polypeptide. The functional consequences of the T9185C mutation have been previously shown in *E. coli* to cause reduction of ATP synthesis. The novel T9191C mutation in patient 2 may have similar functional consequences as the T9185C, since both the mutations are located in the same region of the polypeptide and result in the same type of amino acid substitution (L → P). Our findings expand the spectrum of mutations causing LS and furthermore emphasize the role of ATPase 6 gene mutations in pathogenesis of LS.

P-175
Measurement of ATP synthesis in fibroblasts from patients with mitochondrial disorders

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Decreased ATP synthesis in fibroblasts is considered one of several diagnostic criteria for respiratory chain (RC) disorders. To investigate the diagnostic value of this functional assay, we have measured ATP synthesis rates in fibroblasts from patients with suspected or proven RC deficiency or pyruvate dehydrogenase (PDH) E1-alpha deficiency. The rates of ATP synthesis were determined in digitonin-permeabilized cells using pyruvate + malate, glutamate + malate and succinate as substrates. Fibroblasts from patients with isolated cytochrome *c* oxidase deficiency had significantly lower ATP synthesis rates with all substrates compared to the controls. PDH-deficient fibroblasts had decreased ATP synthesis rates with pyruvate + malate when expressed relative to the rates with glutamate + malate or succinate. Fibroblasts from patients with other isolated or combined RC deficiencies showed more variable results; some had low ATP synthesis rates and others had normal rates. In a few cases, decreased ATP synthesis rates in fibroblasts were found in patients with normal RC enzyme activities. Our data confirm the usefulness of the ATP production assay as a supplementary diagnostic tool for mitochondrial disorders.

P-176
Heart transplantation in a patient harboring the mtDNA 3271T>C mutation

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Dysfunction of mitochondria can affect any organ system, though tissues such as mucle, heart, and brain are more susceptible to oxidative phosphorylation (OXPHOS) defects because of their high-energy requirement.

We identified the 3271T>C mtDNA mutation (''MELAS mutation'') in the explanted heart from a 15-year-old Portuguese girl presenting with dyspnea and marked asthenia after mild exercise in the past months. She had had echocardiographic features of dilated cardiomyopathy with severe dysfunction of left ventricle which were confirmed upon an endomyocardiac biopsy. The central nervous system was not affected. After cardiac transplantation, she presented generalized tonic-clonic seizures with respiratory arrest. The patient was on a comatous state with frequent episodes of status epilepticus.

Morphological studies and biochemical analyses of OXPHOS activities in skeletal muscle biopsy were normal. Seven months after transplantation, a brain MRI revealed alterations compatible with a mitochondrial disease.

mtDNA analysis in a paraffin-embedded heart fragment showed the heteroplasmic 3271T>C mutation. Molecular studies in blood from five maternal relatives and in muscle from the patient showed different levels of the mutation.

Conclusion: This case reinforces the notion that dilated cardiomyopathy can be the presenting and predominant clinical expression of the mtDNA 3271T>C mutation.

P-177
Directly repeated motifs occur more often than expected in mtDNA

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Rearrangements on the human mtDNA molecules are to blame for a great variety of pathological states that result from the dysfunction of the respiratory chain and the failure to retrieve cellular energy. They are either deletions, duplications or inversions of parts of the molecule within a pair of breakpoints that frequently appear as short, directly repeated (DR) sequences of up to 13 bp. Probable mechanisms for the rearrangements are illegitimate recombination and replication slippage.

We surveyed the CRS mtDNA molecule in search for DR, using the DknSet program. An astonishing value of 158 255 DR from 5 bp onwards was obtained, with relative proportions of: 5 bp—71.570%; 6 bp—21.135%; 7 bp—5.900%; 8 bp—1.695%; 9 bp—0.500%; 10 bp—0.156%; 11 bp—0.031 bp; 12 bp—0.010%; 13 bp—0.003%; 14 bp—0.001%; and 15 bp—0.001%.

We then generated three assays of random sequences with the same size and base composition as the CRS. The mean DR distribution of the random assays was significantly different from the distribution of the CRS ($P = 0.0011$; S.E. = 0.00106), the random sequence harbouring systematically a lower number of DRs (average 143 171). In contrast, comparing the human and the chimpanzee mtDNA sequences, the DR distributions were not significantly different ($P = 0.57502$; S.E. = 0.04510).

Therefore, Homo and Pan sequences carry a significantly higher number of DRs than expected by chance. Furthermore, since all DR motifs, irrespectively of their length, appear to be evenly overrepresented, the excess cannot be attributed to any kind of codon based bias. It is thus more tempting to hypothesise that higher order molecular stringencies relevant to mtDNA spatial structure do shape sequence features of the molecule.

P-178
Reconstitution of the mammalian mitochondrial DNA replisome in vitro

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We here reconstitute the mammalian mitochondrial DNA (mtDNA) replisome in vitro. The mtDNA polymerase (POL?) cannot use double-stranded DNA (dsDNA) as template for DNA synthesis. Similarly, the TWINKLE DNA helicase is unable to unwind longer stretches of dsDNA. In combination, POL? and TWINKLE form a processive replication machinery, which can use dsDNA as template to synthesize single stranded DNA molecules of about 2 kb. The addition of the mitochondrial single-stranded DNA binding protein stimulates the reaction further, generating DNA products of about 16 kb, the size of the mammalian mtDNA molecule. The observed DNA synthesis rate is 180 base pairs per minute, corresponding closely to the previously calculated value of 270 base pairs per minute for in vivo DNA replication. Our findings provide the first biochemical evidence that TWINKLE is the helicase at the mitochondrial DNA replication fork. Furthermore, mutations in TWINKLE and POL? cause autosomal dominant progressive external ophthalmoplegia (adPEO), a disorder associated with deletions in mitochondrial DNA. The functional interactions between TWINKLE and POL? thus explain why mutations in these two proteins cause an identical syndrome.

P-179
Shifting mitochondrial DNA heteroplasmy by mitochondrially targeted restriction endonucleases

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In a heteroplasmic environment, the wild-type mtDNA fraction has a strong protective effect, and cellular defects are only observed in the presence of very high percentages of mutated mtDNA. We have developed a system to decrease specific mtDNA haplotypes by expressing mitochondrially targeted restriction endonucleases in vivo. Such system has the potential to reduce a mutated mtDNA fraction, if a restriction endonuclease site is created by a pathogenic nucleotide alteration. As a model system, we used hepatocytes from heteroplasmic mice harboring mtDNA from both BALB and NZB haplotypes. Among several nucleotide variations, these haplotypes differ by the absence of ApaLI sites in the NZB mtDNA and the presence of a single site in the BALB mtDNA. Transfection of mitochondrially targeted ApaLI in these cells resulted in a rapid shift in heteroplasmy towards the NZB mtDNA. To better understand the timing of heteroplasmy shift, we placed the mitochondrially targeted ApaLI gene in a vector system inducible by mifeprestone (RU486). We have analyzed the kinetics of heteroplasmy shift. Heteroplasmic hepatocyte clones containing the inducible

construct treated with mifeprestone showed a complete shift in heteroplasmy and became homoplasmic for the NZB mtDNA in less than 24 h. We also detected a fast recovery of mtDNA depletion caused by the induction of the system. These results indicate that the heteroplasmy shift triggered by mitochondrially targeted restriction endonucleases is extremely fast and specific. We are currently testing the system in a live animal, using infection with AAV in different tissues of heteroplasmic mice.

P-180

Automatic analysis of variability in the mitochondrial DNA from human cell lines: implications in functional genomics

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Cell lines as experimental models in biology have been used for a long time. Nowadays, is well known that mitochondrial DNA (mtDNA) play an important role in cancer, apoptosis, and neurodegenerative diseases. However, no detailed analysis of variability in the mtDNA of human cell lines used in a routine way as experimental models of these processes has been done.

In this study, the complete mitochondrial genome of 13 human cell lines related to cancer, apoptosis, and neurodegenerative diseases has been analysed. For this purpose, a relatively simple and quick approach for automating the detection of sequence variants in human mtDNA has been developed. This approach involves the simultaneous generation and purification of 24 overlapping PCR fragments of 800–1000 bp length in a multifunctional robot followed by their automatic sequencing. The assembling of sequences and identification of variations has been carried out using specialised software.

Among the 13 cell lines sequenced 186 variations from the Cambridge sequence reference were identified. Five cell lines had no apparent functionally significant mutations, i.e., all found sequence variations were previously described polymorphisms, whereas each of the remaining eight cell lines harboured at least one new variant of the coding sequence. Functional analysis of these variations, currently in progress, suggests impaired oxidative phosphorylation activity.

In summary, we report an automatic approach for quickly detecting and generating mtDNA variability data. In addition, we show that human cell lines present high variability in their mtDNA which might represent a useful tool in the functional genomic analysis of the mitochondrial genome.

P-181

A frameshift mutation leading to an unstable mRNA in a boy with dihydrolipoamide dehydrogenase (E3) deficiency

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Dihydrolipoamide dehydrogenase (E3) is a component of the pyruvate dehydrogenase complex (PDHc), the alpha-ketoglutarate dehydrogenase complex and the branched-chain alpha-keto acid dehydrogenase complex. A boy from non-consanguineous parents was diagnosed with E3 deficiency. He presented at day 4 with hypotonia, metabolic acidosis (pH 6.99, lactate 16.2 mM, elevated lactate/pyruvate ratio), ketonuria, 2-oxoglutaric and 2-OH isovaleric aciduria. Plasma branched-chain amino acid levels were normal. After a transient improvement, a fatal metabolic decompensation occurred at 3 months. Decreased activities of PDHc and E3 were found in fibroblasts (20% and 5% of controls, respectively). Western blot analysis using a specific anti-E3 antibody displayed a barely detectable E3 subunit, comparing to the immunoreactivity of control E3 and all the other PDHc subunits. cDNA sequencing analysis revealed only a homozygous transition, 1174 G>A (V392M) in exon 11. This mutation was found heterozygous in exon 11 genomic DNA and inherited from the father. An unstable mRNA species was then suspected in the patient, corresponding to the other allele. Sequencing of the 14 exons and exon–intron boundaries enabled us to find the second heterozygous mutation: 858_859insT (287fs290X) in exon 9, inherited from the mother. As this mutation created a premature stop codon, a mechanism of nonsense mRNA decay may be suggested to explain the unstability of the corresponding mRNA.

P-182

Cultured cell phenotypes of patients with Twinkle and POLG mutations

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Defects of the new mitochondrial DNA helicase Twinkle and polymerase gamma (PEO-proteins) cause autosomal recessive or dominant progressive external ophthalmoplegia (PEO) associated with various muscle and nervous system symptoms, such as polyneuropathy, depression, ataxia and parkinsonism. Our aim was to determine the functional consequences of PEO-protein defects in cultured human primary fibroblasts and myoblasts, as well as in retroviral-

transduced cell lines expressing mutant and wild-type PEO-proteins.

We studied the levels of the PEO-proteins and their RNAs in native state in control and patient lines, carrying a 39-bp duplication-mutation of Twinkle, as well as dominant POLG lines with Y955C and R953C mutations. We also followed the expression of Twinkle, POLG, POLG2 and other mitochondrial proteins in induced oxidative stress by quantitative PCR. We also studied the viability of the cells in induced oxidative stress and when exposed to DNA alkylating agents. We studied the DNA-repair system of the mitochondria in the patient cells, as well as the consequences of the expression of the mutant proteins.

We present here functional data supporting the role of Twinkle as a replication helicase, as well as data describing the cellular phenotype of mutant Twinkle and polymerase gamma.

P-183

The Harlequin mouse: a faithful mouse model for Human complex I deficiency

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Upon a variety of either physiological or adverse conditions, mitochondria release intermembrane space proteins prone to trigger cellular apoptosis. Besides being pro-apoptotic effectors, these proteins may hold additional and yet apparently unrelated function, e.g. electron transfer for cytochrome *c*. In an attempt to characterize such a potential function for AIF (Apoptosis Inducing Factor) protein, we discover that a variety of conditions where AIF expression is either absent or strongly reduced lead to a severe complex I deficiency and abnormal subunit composition of this latter complex (Vahsen et al., submitted for publication). Two years ago, Klein and colleagues [Nature 419 (2002) 367] showed that the Harlequin mouse has reduced AIF expression due to a proviral insertion in the AIF gene. The insertion resulted in an 80% decreased AIF protein and a simultaneous oxidative stress. AIF-depleted Harlequin mice develop progressive neurological involvement with optic neuropathy and retinal degeneration. We therefore studied respiratory chain activity in the Harlequin mouse, and found profound complex I deficiency in affected tissues. Complex I activity was particularly decreased in the retina, in agreement with the retinal degeneration observed in this mouse. The function of AIF in the biogenesis/maintenance of complex I is still to be clarified. However, the progressive neurological deterioration, with optic involvement, and the late onset cardiomyopathy are strongly reminiscent of the clinical symptoms associated with complex I deficiency in human. This makes Harlequin mouse model the first faithful model for complex I deficiency which will be used to test for therapeutic compounds.

P-184

Screening for mutations in DGUOK in patients with mitochondrial DNA depletion

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Mitochondrial DNA depletion syndrome (MDS) are a group disorders characterised by a reduction in the mtDNA copy number. The myopathic form of MDS has been associated to mutations in the thymidine kinase 2 gene (TK2), and the hepathocerebral form to deoxyguanosine kinase (DGUOK).

As most of our patients presented the hepathocerebral form, we started with the study of DGUOK. We selected 26 patients with the clinical and biochemical characteristics of hepatocerebral MDS. In 18/26, mtDNA depletion was demonstrated in one or more tissues by Southern blot. DGUOK was studied in genomic DNA by PCR and sequencing.

We have found mutations in three families. Patient 1 was homozygous for 763dupGATT, a mutation previously reported by Salviati et al. (2002). In addition, we have identified a new mutation which is present in homozygous form in four patients of two unrelated families. The new mutation (c677 A>G) changes histidine 226 to arginine (H226R). It is present in heterozygous form in the parents, but was absent in 100 control alleles. Histidine 226 is highly conserved between species and between different deoxynucleotide kinases; and its substitution for arginine would provoke a change in the hydrophobicity leading to incorrect conformation of the protein.

Our results have important consequences for the affected families, who may now apply for prenatal diagnosis in future pregnancies. Nevertheless, only a part of our patients presented mutations in DGUOK. Therefore, it is necessary to further investigate other genes and mechanisms that may be implicated in the disease.

P-185

Mitochondrial saponin permeabilisation prior to measurement of complex I activity

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In measurements of complex I and complex I + III activities, it is a challenge to sufficiently permeabilise the mitochondrial inner membrane to give NADH full access to its binding site at the matrix side of complex I without disrupting the integrity of the respiratory chain complexes. Common published values for complex I and complex I + III activities in isolated mitochondria range from 0.1 to 0.5 mmol/min/g protein (35 C). These values are apparently low when compared to oxymetric measurements of coupled oxidation of NADH-linked substrates or uncoupled oxidation of NADH, indicating turnover rates in the respiratory chain that are nearly one magnitude higher. A possible reason for the discrepancy is lack of an adequate sample preparation method prior to the measurements of the single enzyme complex activities. Saponin, a detergent extracted from Quillaja bark, was found to effectively permeabilise mitochondria. Unlike other detergents, the integrity of the respiratory chain enzymes appears to be maintained after the saponin treatment. We developed a quick and straightforward preparation procedure resulting in complex I and complex I + III activities that are well in level with the turnover rates derived from oxymetric measurements. The procedure has been used for a while in our laboratory, and has been approved to detect patients with mitochondrial disorders and to deliver reliable results in several investigations of mitochondrial function. In conclusion, the improved preparation procedure leads to a considerable reduction of the sample size needed for the analysis, and probably also to a higher accuracy and precision in the measurements.

P-186

Mitochondrial DNA depletion in asymptomatic HIV-infected patients receiving didanosine plus stavudine-based antiretroviral regimen seems to be compensate by up-regulatory mechanisms

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Objective: The use of nucleoside analogues is uniformly associated with mitochondrial DNA (mtDNA) depletion, but diverse studies in asymptomatic patients have reported functional indemnity of mitochondria. We determined whether homeostatic mechanisms are able to compensate this mtDNA depletion in patients receiving stavudine plus didanosine (d4T + ddI), an antiretroviral association with great in vitro and in vivo capacity to decrease mtDNA.

Design: A prospective, cross-sectional study with cases and controls.

Methods: We included 28 symptom-free HIV-infected individuals: 17 on first-line antiretroviral regimen consisting of d4T + ddI for at least 6 months (case group) and 11 naïve subjects (control group). In peripheral blood mononuclear cells we assessed: (1) the quantity of mitochondria by citrate synthase activity, (2) the content of mtDNA by quantitative real-time PCR, (3) COX-II expression (subunit II of cytochrome *c* oxidase (COX), encoded by mtDNA) by Western blot, and (4) COX activity by spectrophotometry.

Results: The quantity of mitochondria and the mtDNA content of cases (d4T + ddI) were decreased when compared to controls, whether calculated by cells or by mitochondria. The expressions of COX-II and COX activities were similar in cases and controls. The expression of COX-II was found to be constant and independent of the mtDNA content, but was closely related to COX activity.

Conclusions: Decreased mitochondrial mass and mtDNA content are associated with ddI + d4T treatment, but the expression of COX-II and COX activity remains unaltered. These data suggest that, at least during the initial phases of treatment, up-regulatory transcriptional or posttranscriptional mechanisms compensate mtDNA depletion caused by ddI + d4T.

P-187

Novel mt-tRNA mutations present in two patients with sensorineural hearing loss

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In a study of 70 Portuguese patients with sensorineural hearing loss (SNHL), we identified two novel mtDNA mutations.

Case 1: A 12-year-old boy harbors a new 5558A>G mutation in the tRNATrp. The mutation affects the highly conserved nucleotide A49 at the bottom of the T psi C stem. This mutation is strongly suspected of being pathogenic as it probably destabilizes the tRNA secondary structure, compromising either the tRNA affinity for its cognate aminoacyl-tRNA synthetase or its placement at the A-site of the ribosome, during translational decoding. MtDNA haplotyping indicates that this patient belongs to the U haplotype, typical of the Iberian population.

Case 2: A 6-year-old girl harbors the new 5618T>C mutation in the tRNAAla gene affecting the conserved A41 nucleotide in the anticodon stem. This mutation is also potentially pathogenic in that it might destabilize the anticodon arm, and affect the tRNAAla folding, via the loss of the interactions with both the D-arm and the variable arm. Interestingly, this patient also harbors the 10044A>G mutation in the tRNAGly, which is rarely observed ($<0.5\%$) in the normal population.

Both novel mutations were not found in 100 ethnically matched controls.

On the whole, our study suggests that seeking genetic alterations in SNHL patients may add to the variegate array of changes in the mitochondrial genome.

P-188
Replication intermediates of human mtDNA in cell lines

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We have studied mtDNA synthesis in intact cells using saponin permeabilisation followed by pulse labeling with 32P-dTTP [Nucleic Acids Res. 29(2) (2001) E1], restriction digestion and size separation on one-dimensional agarose gels. After Southern blotting the newly synthesized mtDNA in each band can be quantitated using a phosphorimager. Corresponding unlabelled DNA in the same band can be quantitated after probing with a mtDNA probe.

We detected transient mitochondrial DNA (mtDNA) forms in pulse-labeled mtDNA samples, which were not readily detectable in unlabelled cells. Analysis of these forms using restriction enzyme and S1 digestion suggests that these species represent paused replication intermediates. These results have implications for the mechanism(s) of human mtDNA replication [J. Biol. Chem. 278(51) (2003)50961; Trends Biochem. Sci. 28(7) (2003) 357].

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P-189
Mitochondrial oxidative capacity in the atrophied rat skeletal muscle: effect of acetyl-L-carnitine

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The impact of acetyl-L-carnitine (ALCAR) administration on the expression profile of atrophying rat soleus muscle as induced by 14 days of hindlimb suspension was investigated. ALCAR was provided ad libitum to a group of control and hindlimb suspended animals ($n=7$) and ALCAR-untreated control and suspended groups were housed under the same condition. ALCAR effects were monitored through a microarray approach using commercially available ATLASTM Rat 1.2 cDNA filters. The statistical method to detect differentially expressed genes relied on L1 regression on all possible permutations of scatter plots of raw values combined with the conservative Bonferroni method to correct the type I error. Statistical significance was assessed at $P<0.05$.

No difference in soleus weight normalized to body mass was noted between ALCAR-treated and untreated groups. Both hindlimb suspended groups underwent a drop in the soleus muscle (-21%) compared to the respective control groups. ALCAR was very effective on fatty acids transport and oxidative metabolism. The decrease in mitochondrial muscle carnitine palmitoyltransferase I isoform with the unloading was prevented with ALCAR dietary. As far as the oxidative phosphorylation metabolism, both nuclear (COX IV, Vb, VIII-H) and mitochondrial (COX I) cytochrome oxidase subunits were up-regulated with nutritional intervention. The results indicate that nutraceuticals administration to unloaded atrophied rat muscle is able to modulate the expression of genes involved in mitochondrial oxidative capacity, improving the fatty acid import as well as the cytochrome oxidase subunits. These findings could suggest a potential role of ALCAR in the therapeutical treatment of muscle disuse conditions as bed rest, immobilization or aging itself.

P-190
Entire mtDNA sequence analysis in Russian LHON family

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Introduction: Leber's Hereditary Optic Neuropathy (LHON) is a mitochondrial genetic disease that preferentially causes blindness in young adult males. It is characterized by acute or subacute loss of central vision due to focal degeneration of the retinal ganglion cell layer and optic nerve. More than 30 mutations of mitochondrial DNA have been associated with LHON patients but only three ''primary'' mutations may contribute in a major way to the development of blindness: G3460A, G11778A, T14484C. Other ''secondary'' mutation may contribute in LHON by increasing the probability of expressing the phenotype.

Patients and methods: We analyzed mtDNA of two patients—mother and son which have the primary mutation T14484C. DNA was purified from blood by standard methods. Entire mtDNA was sequenced after long-range PCR followed by automated sequencer (Beckman-Coulter CEQ8000). After we have sequence information, we evaluated by PCR-RFLP analysis.

Results: We found following secondary mutations T4216C, G13708A, and G15812A of mtDNA in both mother and son. However, we found additional secondary mutation G15257A of mtDNA in mother. Majority of nonpathogenic polymorphism have seen in both patients.

Conclusion: According to our sequencing date, we may suggest that mtDNA is not always transmitted completely in disease state.

P-191
Mitochondrial DNA mutations in ragged red fibers of healthy individuals of different age

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In order to get insights on the mechanisms of formation of ragged red fibers in human skeletal muscle during aging, we analyzed the mitochondrial genotype of single skeletal muscle fibers of 15 healthy individuals having an age comprised between 45 and 92 years. The sequencing of the D-loop region showed many sequence changes with respect to the Cambridge Reference Sequence, in both RRF and normal fibers. These changes were more abundant in RRF and their number increased between 50–60 and 60–70 years and then remained approximately constant. Each subject contained one or more changes associated to RRF in positions of D-loop region that either do not change or that change very rarely. In general, the same type of RRF-associated change was not found in more than one individual; exceptions were changes in positions 189, 295, 374 and 514, detected in 20–50% of analyzed subjects. In particular, the A189G age-associated mutation was found only in old individuals and prevalently in RRF. Sequencing of other two mtDNA regions showed no relevant changes in the 16S/ND1 region and two RRF-associated original mutations, G5847A and A5884C, in two very conserved positions of tRNATyr. These results indicate that each subject has its own pattern of RRF-associated sequence changes in both coding and non-coding region of human mtDNA. Mutations in the coding region might impair mitochondrial function, whereas changes in the D-loop might affect mtDNA replication rate and might produce large-scale deletions due to strand slippage during mtDNA replication of mutated molecules.

P-192
Presence of complex V subcomplexes in the patients with oxphos defects

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The oxidative phosphorylation (OXPHOS) generates ATP through passage of electrons along the four complexes of the respiratory chain (I–IV) in the mitochondrial inner membrane. ATP synthase (complex V) is a multisubunit enzyme consisting of a catalytic portion (F1), a membrane portion (F0) and two stalks linking F1 and F0. Using Blue Native Polyacrylamide Gel Electrophoresis and subsequent catalytic staining for ATPase, only one band corresponding to complex V was detected in most of the patients. In nine patients, however, in addition to the normal band, complex V subcomplexes with lower molecular weight were detected. This phenomenon was seen in skeletal muscle (two patients), liver (three patients) and in cultured skin fibroblasts (four patients). A severe, combined deficiency of OXPHOS complexes (I, III, IV and V) together with normal activity of complex II was seen in two patients, and a less severe, combined deficiency (I and IV) in four patients. Two of the latter patients were carriers of a MERRF point mutation. In three patients no other defects of OXPHOS complexes were found. The exact cause of incomplete complex V synthesis, or decreased stability, is not known. One likely hypothesis is a defective intramitochondrial protein synthesis. The associated finding of a combined OXPHOS complex deficiency in several patients is in favor of this hypothesis. Remarkably, two of these patients carried a MERRF mutation. Other possible explanations are a mutation in a complex V subunit, especially in subunit 6 or increased intramitochondrial oxidative stress.

P-193
Action of carnitine in treatment of patients with glutaric aciduria type 1

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Glutaric aciduria type I is caused by the deficiency of glutaryl-CoA dehydrogenase in the mitochondria. In the past, most children with glutaric aciduria type 1 suffered from a sudden severe neurological disease and died before the age of 10 years. Since the introduction of carnitine and the limitation in the dietary intake of lysine and tryptophane in treatment of the children, they develop normally, provided the treatment is instituted before the encephalopathy occurs. The pathogenesis of this disease is still not fully understood. There are several hypotheses explaining the brain disease in the condition.

We studied the possibility that glutaryl-CoA could act as an inhibitor of 2-ketoglutarate dehydrogenase of the Krebs cycle, and that this inhibition is abolished by carnitine. This hypothesis was supported by the finding that 25 μM glutaryl-CoA inhibited 2-ketoglutarate dehydrogenase (from 100% to 54%) in homogenate from cultured fibroblasts from a patient with glutaryl-CoA dehydrogenase deficiency, and not in control fibroblasts (100% to 93%). The inhibition in the patient cells was abolished by L-carnitine (54% to 89%).

P-194
Cells lacking mitochondrial DNA as a model to study the interaction between the respiratory chain and cellular proliferation

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We have used HeLa rho0 cells to analyze how the absence of a functional respiratory chain slows down proliferation. ATP content and energy charge were the same in wt and rho0 cells, ruling out these parameters as important causes for the proliferative defect of rho0 cells. Flow cytometric analysis revealed that the distribution of cells in different stages of the cell cycle was not different in wt and rho0 cells, implying that a deceleration of all stages was responsible for the growth defect of rho0 cells. Cell cycle progression is under control of cell cycle-dependent kinases in association with specific cyclins, some of which were analyzed by Western blotting. Expression of cyclin E and cyclin A, which control initiation and progression of S-phase, and cyclin B, which controls G2/M, were not different in both cell lines, consistent with similar cell cycle profiles. However, we found global decreases in the expression of kinase inhibitors (p18, p19, p21CIP1/WAF, p27), which control D-type specific kinase activity as well as down-regulation of cyclin D3 protein expression. In summary, our data suggest that respiratory chain activity influences cellular proliferation by as yet unknown mechanisms other than general energy supply.

P-195
Respiratory complex III is required to maintain complex I in mammalian mitochondria

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A puzzling observation in patients with oxidative phosphorylation (OXPHOS) deficiencies is the presence of combined enzyme complex defects associated with a genetic alteration in only one protein-coding gene. In particular, mutations in the mtDNA encoded cytochrome *b* gene are associated either with combined complex I + III deficiency or with only complex III deficiency. We have reproduced the combined complex I + III defect in mouse and human cultured cell models harboring cytochrome *b* mutations. In both, complex III assembly is impeded and causes a severe reduction in the amount of complex I, not observed when complex III activity was pharmacologically inhibited. Metabolic labeling in mouse cells revealed that complex I was assembled, although its stability was severely hampered. Conversely, complex III stability was not influenced by the absence of complex I. This structure dependence among complexes I and III was confirmed in a muscle biopsy of a patient harboring a nonsense cytochrome *b* mutation.

P-196
Mitochondrial DNA adaptative mutations affect sperm motility and latitude distribution

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We have proposed that as humans migrated out of Africa into temperate Eurasia, new mutations were selected in the mitochondrial DNA (mtDNA) that shifted the energy balance (coupling efficiency) of oxidative phosphorylation (OXPHOS) from primarily ATP production to increased heat production. To test this hypothesis, we have analysed the mtDNAs of the ancient and diverse European haplogroup U for mutations that could increase heat and decreased ATP production. Three such lineages were identified harbouring four different cytochrome *b* (cytb) mutations. To determine if these cytb mutations caused reduced ATP production, we examined the motility rate of sperm from each U sub-lineage and found that lineages with founding cytb mutations had reduced sperm motility. To determine if these same lineages had increased heat production, we examined the north–south European distribution of the U sub-lineages and found those with cytb mutations were enriched in the more northern European latitudes. Thus, haplogroup U sub-lineages with founding cytb mutation have the features expected for cold-adaptive uncoupling mutations.

P-197
Familial mitochondrial myopathy and diabetes mellitus due to a rare mtDNA mutation (tRNAGlu 14.709T>C): clinical presentation and therapeutical effects

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Background: Pure mitochondrial myopathies (MM) are frequent in adult but rare in childhood patients, and present with muscle weakness, hypotonia, exercise intolerance and external ophthalmoplegia. ‘‘Ragged red fibers‘‘ (RRF) and combined complex I + IV deficiencies are the morphological and biochemical hallmarks in muscle biopsies. A wide range of mitochondrial (mt)DNA mutations have been described which usually involve tRNA genes of the mitochondrial genome (mtDNA).

We here describe a family in whom the mother and her daughter present with typical clinical signs of MM and in which the disease segregates with a rare mt-tRNA mutation.

Goals of this study: To describe the clinical, morphological, biochemical and genetic presentation of MM in a family and the effectiveness of various therapeutical attempts.

Case report and results: The 15-year-old girl showed hypotonia and delayed motor milestones since early infancy and developed exercise intolerance with crampi and diabetes mellitus during the following years. The 43-year-old mother complains about increasingly exercise intolerance and muscle crampi since some years. In both, numerous RRF and a combined complex I + IV deficiency were found on muscle biopsy. Sequencing of all mt-tRNA genes in muscle DNA revealed a heteroplasmic T-to-C transition at position 14.709 in the tRNAGlu gene with higher levels in the daughter than in the mother. A therapeutical trial with riboflavine and creatine monohydrate was of great benefit in the daughter—as indicated by bicycle ergometry—but not in the mother.

Conclusion: We describe a family with the rare mt-tRNAGlu mutation 14.709T>C. This maternal inherited mutation manifests as a pure MM, which can successfully be influenced by cofactors of the energy metabolism, and as diabetes mellitus.

P-198
Homozygous POLG mutation in a young patient presenting with SANDO phenotype

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POLG mutations in PEO families compatible with recessive inheritance have been previously reported. Here, we describe a 15-year-old boy who first presented with frequent falls secondary to a cerebellar ataxia. The family history is not contributory and he has a healthy younger sister. The other clinical features of ophthalmoplegia and dysarthria led to the clinical diagnosis of sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO). Histopathological examination revealed typical mitochondrial myopathy features with 30% of ragged fibers. Biochemical analysis showed decreased activities in complexes I, III and IV. Multiple mtDNA deletions were found in muscle. We identified an L304R homozygous POLG mutation in exon 4. This mutation has been previously described in a Belgian SANDO family, carrying compound heterozygous POLG mutations. Onset age was 8 years in our patient, 16–25 years in Belgian patients who had a milder phenotype. This observation demonstrates that homozygous POLG mutations can be responsible for ArPEO patients with SANDO phenotype.

P-199
Exertional dyspnea in mitochondrial myopathy: a consequence of severely impaired muscle oxidative phosphorylation

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Exertional dyspnea (ED), mimicking severe cardiopulmonary disease, may be the dominant symptom of mitochondrial myopathy (MM). We investigated this symptom by comparing responses to peak cycle exercise (ventilation, VE; oxygen uptake, VO2; carbon dioxide production, VCO2; ventilatory equivalent for O2, VE/VO2; respiratory exchange ration, VCO2/VO2; cardiac output, Q; and a-v O2difference—calculated from the Fick equation, VO2 = Q × a-v O2difference) in six patients with prominent ED (EDMM), compared to 30 patients in whom dyspnea was less prominent (MM) and 30 healthy subjects (HS). Resting values were similar in all subjects. Peak VO2 in exercise in EDMM (10.2 + 2.6 ml/kg/min) and MM (17.7 + 8 ml/kg/min) were lower than controls (32.3 + 7.0 ml/kg/min), due to a low peak a-vO2diff (EDMM = 4.1 + 1.1, MM = 8.8 + 3.4 ml/dl, controls = 15.22 + 2.1 ml/dl). Peak VE in EDMM (69 + 26 l/min) and MM (58 + 24 l/min) were lower than in HS (103 + 32 l/min) and peak VE/VO2 was dramatically higher in EDMM (101 + 20) compared to MM (58 + 16) and HS (41 + 7). Similarly peak RER was higher in EDMM (1.66 + 0.4) than MM (1.18 + 0.01) or HS (1.14 + 0.06). In both patient groups, peak VE/VO2 showed a close inverse relationship ($r^2 = 0.73$) to the level of impaired oxidative phosphorylation, as reflected in peak a-vO2diff. We conclude that prominent ED in MM is associated with markedly exaggerated ventilation and carbon dioxide production in relation to O2 utilization. The degree of hyperventilation correlates inversely with the level of muscle oxidative defect, consistent with the hypothesis that exertional dyspnea is a direct consequence of the severity of impaired oxidative phosphorylation in mitochondrial myopathy.

P-200
Point mutations of mtDNA associated with acute leukemia developing from myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) are clonal myeloid disorders characterized by ineffective hematopoiesis resulting in refractory cytopenias. Transformation resulting in acute myeloblastic leukemia is the final stage in the multistep process of MDS evolution. The MDS subtype sideroblastic anemia has been shown to be associated with mutations of mtDNA. To search for alterations of the mitochondrial genome in other subtypes of MDS and leukemia, we used high-resolution techniques like SSCP and fluorescent sequencing for investigation of the whole mitochondrial genome of 10 patients with MDS. New point mutations, fulfilling the criteria for pathogenetically relevant mtDNA alterations in $tRNA^{Tyr}$, $tRNA^{His}$, 16S rRNA, and ATP synthase subunit 6, were detected. Our work shows that mutations of the mitochondrial genome exist not only in sideroblastic anemia but also in other subtypes of MDS. MDS and the developing acute myeloid leukemia are connected by an increasing mutation load of mtDNA. Several point mutations of mtDNA in the bone marrow of one patient, including secondary point mutations for Leber's hereditary optic neuropathy (LHON), occur in one bone marrow and may synergically affect bone marrow stem cells by an apoptotic pathway.

P-201
In utero exposure to anti-HIV drugs causes mitochondrial damage and mtDNA depletion in umbilical cord tissue from human infants and fetal Erythrocebus Patas monkeys

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Nucleoside reverse transcriptase inhibitors (NRTIs), when given to HIV-1-infected pregnant women, effectively reduce the risk of mother-to-child HIV transmission. Mitochondrial dysfunction, a toxic consequence of NRTI exposure in adults, has been reported in a few HIV-1-uninfected children born to women receiving AZT and/or 3TC during pregnancy. However, most exposed children do not present with clinical symptoms. To identify potential molecular mitochondrial damage in the fetus, we evaluated mitochondrial integrity in umbilical cords from HIV-1-uninfected infants born to HIV-1-infected women receiving Combivir (AZT + 3TC) during pregnancy, and in fetal Erythrocebus Patas monkeys born to HIV-1-uninfected dams given 3TC, AZT + 3TC, AZT + ddI or

3TC + d4T during gestation. Umbilical cords from offspring of HIV-1-uninfected mothers and Patas dams were used as controls. Mitochondrial morphology was analyzed by electron microscopy, and mtDNA was measured by hybrid capture chemiluminescence assay. Severe mitochondrial ultrastructural pathology, characterized by cristae dissolution and membrane disruption, correlated ($r = 0.939$) with significant mtDNA depletion (27–78%) in umbilical cords from exposed human infants and fetal monkeys. In the monkey model, the degree of morphological damage due to NRTI exposure ranked as follows: 3TC + d4T>AZT + ddI>AZT + 3TC>3TC. Despite the fact that the infants were born to HIV-1-infected mothers and the fetal monkeys were born to HIV-1-uninfected dams, strikingly similar mitochondrial damage was observed in the exposed offspring. This indicates that NRTI treatment alone, irrespective of HIV-1 status, can cause mitochondrial toxicity.

P-202
Assessment of oxidative stress in transmitochondrial cell lines

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The oxidative phosphorylation (OXPHOS) system is the major production source of reactive oxygen species (ROS) in cells. It is thought that oxidative stress could play an important role modulating the cell phenotype produced by mtDNA mutations. The aim of the study was to assess the status of free radical production and damage in cybrid cell lines harbouring different mtDNA point mutations. These included the A8344G mutation in tRNALys (MERRF line), the A3243G mutation in tRNALeu(UUR) (MELAS line), and the G6930A mutation in COX-I (COX line). Biochemical studies to assess the levels of free radical damage and antioxidant defences included: hydrogen peroxide production, superoxide dismutase (SOD), catalase and glutathione peroxidase activities. Western blot analysis of SOD and catalase proteins was performed to evaluate a putative increase in protein levels. Lipid peroxidation was assessed by measuring the malondialdehyde levels.

Hydrogen peroxide production and antioxidant enzyme activities were increased in MERRF and MELAS cell lines, but no increase was observed in COX cell line. Western blot analysis suggested a different pattern for SOD and catalase proteins (increase in SOD and maintenance in catalase levels). Any of these cell lines showed changes in lipid peroxidation levels.

These results suggest that the impairment in the activity of the OXPHOS system (such as in MELAS and MERRF cells) leads to an increase in ROS production. This increase results in an up-regulation of the antioxidant system. Oxidative stress status will be more important in some mutations than in others, and probably would modulate the phenotype of some mtDNA mutations.

P-203
Maternal inheritance in cyclic vomiting syndrome

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Background: Cyclic vomiting syndrome (CVS), characterized by severe discrete episodes of nausea, vomiting and lethargy, is a fairly common and disabling pediatric condition that is associated with dysautonomia. Our group recently published that maternal inheritance is present in most children with CVS and additional neuromuscular disease manifestations.

Methods: To determine if CVS in general demonstrates maternal inheritance, a structured clinical interview was administered to both parents of 80 unrelated individuals with CVS ascertained randomly from the database of the Cyclic Vomiting Syndrome Association. HV1 and HV2 were cyclosequenced in patients and in 95 controls.

Results: Subjects and their matrilineal relatives suffer at a far higher incidence from depression, hypothyroidism, and several dysautonomic-related conditions including migraine, irritable bowel and chronic fatigue. There was a strong bias for maternal inheritance of disease, including in mothers vs. fathers (relative risk 5.5; $P = 3 \times 10^{-9}$) and in maternal versus paternal grandmothers (relative risk 4.0; $P = 2 \times 10^{-6}$). Maternal inheritance is suggested in 53% of the families, with no difference between subjects with and without neuromuscular disease. Single nucleotide polymorphisms were threefold ($P < 0.01$) more frequent in CVS than in control subjects in an evolutionarily highly conserved 150-bp region of HV1 encompassing TAS.

Conclusions: Our data strongly suggest that mtDNA sequences predispose towards the development of disease in our CVS subjects and their matrilineal relatives, and that CVS is but one potential clinical presentation. Our findings have important applications in the treatment and counseling of individuals with CVS.

P-204
Modifiers of mitochondrial disease phenotype in *Drosophila*

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Human mitochondrial disease manifests with a wide range of clinical phenotypes of varying severity. We have manipulated the *Drosophila* gene technical knockout (tko), encoding mitoribosomal protein S12, in order to create an animal model to study some of these disorders.

A point mutation in this *Drosophila* gene (tko) produces distinct developmental phenotypes of relevance to mitochondrial disease. The developmental and pathophysiological abnormalities associated with the tko mutant, L85H, are being studied using the GAL4 conditional expression system. The wild-type tko gene has been engineered in to a P-element construct that places it under the control of the GAL4 UAS. Three UAS-tko+ lines have been selected for detailed phenotypic characterization. Selective expression of the wild-type allele indicates critical times and cell types in development, in which mitochondrial protein synthesis deficiency leads to specific phenotypic outcomes.

Currently, I am employing in situ hybridisation and real time PCR to localise and quantify the specific tko mRNA sequences in the three UAS-tko+ lines both with and without induction by exogenously supplied Gal4p. The three genes affected due to the transgene's placement in the genome will also be studied.

The third instar larvae stage is the major growth period during larval development and we have previously shown that restoration of mitochondrial translational capacity at this stage is sufficient to overcome almost half of the tko25t developmental delay.

In the adult fly, the bang-sensitive phenotype is essentially the result of a neural deficit of mitochondrial bioenergy, suggesting the involvement of the nervous system in mitochondrial translation disorders.

P-205
Spontaneous recovery of a childhood-onset mitochondrial myopathy caused by a stop mutation in the mitochondrial cytochrome *c* oxidase III gene

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Recently, we reported on a patient suffering from mitochondrial myopathy with ragged red fibers (RRF), lactic acidosis, exercise intolerance and delayed growth with a heteroplasmic G9379A nonsense mutation (W58X) in the mtDNA encoded COIII subunit gene.

An actual follow-up examination of the patient showed remarkable clinical and electrophysiological improvement. On a second muscle biopsy, signs of histological and immunohistological improvement of the mitochondrial myopathy were found, which was associated with a significant decrease (from 93% to 50%) of the mutational load of G9379A in skeletal muscle confirming a spontaneous regression of the disease. Myoblasts of the patient did not carry the mutation, therefore, we suggest that the fusion of wild-type mtDNA containing myoblasts into existing muscle fibers might positively influence the mutational rate in our patient's muscle.

Our results demonstrate the variable course of diseases caused by mtDNA mutations. We suggest that this possible positive outcome should be considered in counselling patients with mtDNA-mediated disorders.

P-206
Protein import pathways to the mitochondrial inner membrane

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In the general protein import pathway of mitochondrial proteins imported from the cytosol, the Tim17p/Tim23p complex of the inner membrane with a matrix-located ATP-driven import motor consisting of Tim44p, mhsp70, and mGrpE mediates import into the matrix. However, mitochondria have a separate TIM machinery for importing polytopic inner membrane proteins including the multispanning metabolite carriers. This system consists of at least two distinct complexes: a 70-kDa complex of Tim9p and Tim10p in the intermembrane space and a 300-kDa inner membrane complex with Tim12p, Tim18p Tim22p, Tim54p, and a fraction of Tim9p and Tim10p. The Tim9p/Tim10p complex guides the carrier proteins across the intermembrane space and the 300-kDa inner membrane complex mediates insertion of the carriers into the inner membrane. Tim9p, Tim10, and Tim12p along with Tim8p and Tim13p form the small Tim family that share an identity of 25% and have conserved cysteine residues. The mitochondrion thus has developed an elaborate import system for guiding the hydrophobic inner membrane proteins across the intermembrane space and mediating insertion into the inner membrane.

P-207
Organization of mtDNA heteroplasmy examined by in situ hybridization

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The existence of multiple forms of mitochondrial DNA (mtDNA) within individual tissues and cells has been shown to be an important determinant of respiratory phenotype and clinical presentation. Both point mutations and deletions of mtDNA are frequently found to exist alongside wild-type (WT) mtDNAs in tissue and cell samples from mitochondrial disease patients. Standard Southern blot analysis of mitochondrial genotype in cultured human cells can efficiently determine the mtDNA species present, but this method cannot distinguish between intercellular heteroplasmy (i.e. a mixture of completely WT and completely mutant cells) and intracellular heteroplasmy, in which WT and mutant mtDNAs coexist within the same cell. We show that two-color fluorescence in situ hybridization (FISH) can distinguish between these two models of heteroplasmic organization and allows sequence-specific determination of mtDNA content and distribution in a cell culture system. Further studies of mtDNA heteroplasmy will benefit from detailed information on the organization of mtDNA genotypes within cultured cell populations.

P-208
Microarray analysis of gene expression in human 143B and A549 cells lacking mitochondrial DNA

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Mitochondrial biogenesis is under the control of two different genetic systems: the nuclear and the mitochondrial genome (mtDNA). To investigate the genetic mechanisms controlling the cross talk between nucleus and mitochondria, we studied the transcriptome of two human cell lines, 143B.206 and A549, which had been entirely depleted of mtDNA (r^o cells), and compared it with the corresponding r^+ parental cells. Affymetrix Microchips U133A, containing 23000 annotated genes, were probed in triplicate with cRNAs extracted from r^o and parental cells. A list of 191 differentially expressed genes, common to both cell lines, was obtained by analyzing the hybridization profiles with dedicated softwares (Mas 5 and Gene Spring). Real-time quantitative reverse transcription PCR (qRT-PCR) is in progress to validate the microarray results.

Only 16 out of the 191 differentially expressed genes are known to encode proteins targeted to mitochondria. However, softwares scoring the likelihood of protein sequences to be targeted to mitochondria (MitoProt, Predotar, TargetP) gave high values for 13 functionally unknown additional proteins, which will be further analyzed by using standard in organulum import assays. Other differentially expressed proteins belong to different metabolic pathways, including purine/pyrimidine biosynthesis, cell cycle, and, notably, response to oxidative stress and apoptosis.

P-209
CIA30 chaperones complex I assembly via several intermediates which relative distribution alters in complex I deficient patients

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Many deficiencies in complex I, the first and largest enzyme complex in the oxidative phosphorylation chain, cannot be explained by means of genetic screening. Since in these cases no mutation is found in any of the genes encoding its 46 subunits, the problem may, analogous to complexes III and IV, be in assembly. Although not much is known about complex I assembly, intensified research in the area has recently led to the first models in humans. Two putative assembly factors are found in the fungus *Neurospora crassa* of which the homologue of one, CIA30, is found in humans. CIA30 is thought to be a chaperone in assembly through the association of the small and large membrane arm assembly intermediates of complex I. Further investigation of its function in human complex I assembly may aid the molecular characterisation of the assembly pathway and thus of many of the unexplained complex I deficiencies.

In this study we have developed an inducible expression system for assembly factor CIA30. Using this system, we show that CIA30 is targeted to the mitochondrion. By using an antibody in combination with two-dimensional blue native/SDS electrophoresis we demonstrate that CIA30 occurs in high molecular weight subcomplexes. The exact composition of these subcomplexes is still under investigation. Interestingly, complex I deficient patients show a significant change in the amount and relative distribution of the CIA30 containing subcomplexes, confirming the important role of this protein in complex I assembly.

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