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THEME 2 GENETICS AND EPIDEMIOLOGY

P16 THE SOD1 TRANSGENE IN THE G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS LIES ON DISTAL MOUSE CHROMOSOME 12

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Background: The SOD1 G93A transgenic mouse strain is a widely studied model of amyotrophic lateral sclerosis (1). These mice carry a human mutant Cu/Zn superoxide dismutase transgene array and have been used in many breeding experiments to look for interactions with other loci, including transgenic and gene targeted mutations. The transgene insertion site is pivotal as this may affect the outcome of such breeding experiments.

Objective: To map the SOD1 transgene in the G93A mouse model of amyotrophic lateral sclerosis by fluorescence *in situ* hybridization (FISH).

Methods: Metaphase spreads were made from SOD1 G93A bone marrow. Plasmid pHG-SOD1^{WT}, containing a human wild-type SOD1 genomic insert (2), was used as a probe to detect the human transgene array in a FISH experiment.

Results: By analysing more than 20 metaphase spreads in the FISH mapping experiment, we determined that the SOD1 G93A transgene insertion site lies on distal mouse chromosome 12. The 'Legs at odd angles' (*Loa*) locus, which is an entirely unrelated mutation in the dynein 1 heavy chain 1 gene (*Dync1h1*), also maps to this chromosome (3). Analysis of a SOD1 G93A x *Loa* cross determined that the site of the transgene insertion lies proximal of the *Dync1h1* gene, on mouse chromosome 12.

Conclusions: We have mapped the SOD1 G93A transgene array to mouse chromosome 12 in band E, by FISH. By analysing a small data set from an existing cross we find the *Loa* mutation in the *Dync1h1* gene lies roughly 31cM distal in what is thought to be band F2. The exact correlation between bands and genetic and physical distances remains to be determined, but it is clear that the two loci are linked, although a considerable distance apart.

The transgene mapping data are important for our SOD1 G93A x *Loa* cross, as knowledge that the two loci are linked now explains the low number of wild-type mice seen in our *Loa* SOD1 x *Loa*+ cross. The position of the transgene array is also relevant to other laboratories carrying out crosses with genes/transgenes of interest on mouse chromosome 12. We believe it would be of interest to the ALS community to determine the site of transgene insertion for other SOD1 mutations.

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P17 UP-REGULATION OF GENES IN MURINE SPINAL CORD TRANSCRIPTOME

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Background: Analysis of the transcriptome in motor neurons, the cells traditionally associated with amyotrophic lateral sclerosis, is hampered both by the diversity of the cell types in nervous tissue as well as the cloaking of the neural elements by the overwhelming glial matrix surrounding these cells. Using a combination of suppression subtractive hybridization (SSH), mirror orientation subtraction (MOS) and normalization of cDNA libraries from spinal cord materials with common glial transcriptome elements, we have constructed a modified library of differentially expressed genes that appear to be up-regulated in spinal cord.

Methods: A differentially expressed cDNA library from murine tissue was constructed using a combination of SSH, MOS and normalization against common glial cDNA sequences. Differential screening using dot blot arrays of clones from these libraries were probed with cDNA derived from forward and reverse subtracted spinal cord and visual cortical cDNA. Positive clones from these screened libraries are confirmed with RT-PCR using Islet-1 (a motor neuron marker) and neuron specific enolase as positive controls.

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Results: One hundred and sixty clones, 20% of 800 randomly selected clones derived from the forward subtracted libraries, were up-regulated in spinal cord tissue when cDNA from spinal cord was used as the probe. Using RT-PCR, we have confirmed five clones that are moderately differentially expressed (1 to 1.5-fold increased), one clone that is differentially expressed (1.5 to 2-fold increased) and one clone that is strongly differentially expressed (greater than 2-fold increased).

Discussion: Sequencing analysis of the cDNA derived from these clones shows a mixture of genes with known and unknown function expressed in the spinal cord compared with visual cerebral cortex. Final confirmation of differential expression by *in situ* and northern blot hybridization and analysis of the possible roles of the proteins produced from these genes and their significance in motor neuron function is ongoing.

P18 A GENOME-WIDE ASSOCIATION STUDY IN SPORADIC ALS USING DNA MICROARRAYS

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Background: Most cases of ALS occur sporadically, however twin studies suggest that the genetic contribution to disease development may be up to 85%. Previous association studies have targeted candidate genes but have yielded few positive, reproducible associations. We have previously used DNA pooling in a whole-genome association study using microsatellites, but it is time consuming to genotype microsatellites in very large numbers using current technology. DNA microarrays allow the mass genotyping of much larger numbers of SNPs than is feasible with microsatellites, and combining this approach with DNA pooling provides a quick and efficient method of performing a genome-wide screen.

Objectives: To perform a genome-wide screen for susceptibility and phenotype modifier genes for sporadic amyotrophic lateral sclerosis (ALS), using DNA pools genotyped on Affymetrix GeneChip© microarrays.

Methods: Cases were selected and matched for sex and age within one year. Pools were genotyped on the Affymetrix GeneChip© 10K Mapping microarrays. Individuals were also genotyped on the microarrays to produce correction factors which adjust the data for differential hybridization.

Results: Pools were constructed of 300 cases and 300 matching controls. Thirty-three individuals were genotyped for data correction. Each pool was hybridized to the microarray in triplicate and median values used for analysis. 11473 SNPs were analysable. Correction factors were available for 9990 SNPs. Using a modified χ^2 which takes measurement error into account, 582 markers were significant at $p<0.05$, and 39 were significant at $p<10^{-7}$. The largest effect sizes were seen for SNPs on chromosome 7 (OR = 15.9, $p<10^{-11}$) and chromosome 3 (OR = 12.3, $p<10^{-18}$).

Discussion and conclusions: DNA microarrays provide the high-throughput technology which genome-wide association studies require. This initial work shows that genotyping DNA pools on microarrays is a useful method for first-pass screening which has quickly and efficiently identified regions associated with ALS to target with fine-mapping studies.

P19 HAPLOTYPE STUDIES IN SMALL ALS FAMILIES: USEFULNESS IN THE SEARCH FOR NEW GENES CAUSING ALS

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Background: Familial ALS forms the tip of the iceberg of motor neuron disorders. Families with ALS can be a powerful tool for finding new genes and may provide valuable clues to the biology of motor neuron functioning. In a small ALS family, we have excluded all known causes of autosomal dominant ALS by analysing the reconstructed haplotype and propose that this family may be associated with a new locus for familial ALS.

Objectives:

1. To find new genes which singly or in combination cause ALS.
2. To examine ALS families which do not have SOD1 mutations in order to locate additional motor neuron disease-causing genes.
3. To store and bank DNA samples (and transformed lymphocyte cell lines) from our study families for future research.

Method: We have been recruiting ALS families for genetic studies for the past 12 years. Eighty-six families with ALS lack the SOD1 mutation and show autosomal dominant inheritance. These families are being tested for the known loci by linkage and/or haplotype analysis. DNA was extracted from whole blood using the Puregene DNA isolation kit. Genotyping for ALS loci was carried out by PCR amplification with short tandem repeat markers at these loci. For affected, deceased individuals whose DNA samples are unavailable, we have reconstructed the haplotypes with spouse and children's DNA.

Results: One of our large SOD1 negative ALS families has a theoretical linkage score (Zmax) 2.7 on simulation analyses. The results of pairwise analyses between disease phenotype in our family and the ALS loci yielded

negative 2-point LOD scores at recombination factor 0; however, the results are not significant to exclude these loci. Using manually reconstructed haplotypes in this family we have been able to exclude all four loci for autosomal dominant ALS without associated fronto-temporal dementia.

Conclusion: Genetic analyses in late onset disease conditions with low disease penetrance may not provide conclusive results. This study shows the difficulties associated with such studies, which may be overcome by running more markers or genotyping individuals in the lower generations to build haplotype information for individuals in the older generation. Thus, small families could provide supporting evidence for new loci or to screen for genes that have been found in larger families.

P20 A NATIONAL NETWORK ON FAMILIAL ALS: AN UPDATE ON COLLECTION AND COLLABORATIONS

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Background: Familial ALS (FALS) represents 10 to 20% of ALS cases. However, only a small part is multi-generational. Moreover, in those multigenerational families SOD1 mutations are frequent (50%). This largely limits the potential of linkage studies in ALS. A large recruitment of FALS cases is necessary to allow such studies to be performed.

Objectives: To describe the organisation of a national network on FALS and its ability to identify families for future genetic work, and to present an update of recruitment up to 2004.

Methods: A national network on FALS was created in 2000. The large majority of university centres are involved. DNA is extracted and cell lines are established to allow genetic studies to be performed. Clinical data are prospectively collected: age of onset, site of onset, presence and diffusion of lower and upper motor neuron signs, date of death. The pedigree is established for all FALS case. For each family, SOD1 mutations are searched for at least one member with ALS. When SOD1 mutations are absent, a CRA manages to collect the family.

Results: Since 2000, 150 new families have been identified and the FALS index-case collected. Almost 80% of the families are composed only of 2 ALS cases. The remaining 20% are multigenerational. We identified a total of 18 families with SOD1 mutations: one with recessive D90A, one compound heterozygote and the other with dominant inheritance. In a large family without SOD1 mutations, genome screen allowed to describe a new locus on chromosome 18q. In 2004, 29 new families have been recruited. Four of those families are multigenerational. In two of them, a SOD1 mutation could be found, confirming our previously published results.

Discussion: Multigenerational, dominant FALS cases are rare. The development of a national network is a useful tool to allow new loci to be found. Collaboration with North America (Professor G. Rouleau) is underway for that proposal. Such a network may also allow sib pair studies to be carried out. A specific programme is underway in a large European collaboration on that topic (Dr. C. Shaw, Dr. J. De Belleroche). It remains surprising that such a large number of families are not multi-generational. Are they dominant with low penetrance, recessive or multigenic? A candidate gene approach (Dr. Corcia, Professor Andres) in those cases could give clues to that point.

Acknowledgement: On behalf of the French group on motor neuron ALS research we would like to thank all the physicians who participate in the network. This is an open network; please do not hesitate to contact us with new ideas.

P21 A NOVEL MUTATION (GLU133VAL) IN CU/ZN SUPEROXIDE DISMUTASE CAUSING FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS IN CHINA

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Background and objective: More than 100 mutations in SOD1 gene have been described in ALS (1). Here we present a two-generation family with ALS from Liaoning province, North China, which has a novel mutation in SOD1 gene.

Methods: We studied the family including four ALS patients and 50 unrelated healthy controls. Exons 1–5 of the SOD1 gene were amplified, the fragments were gel purified and sequence analysis performed.

Results: The proband is a member of a family of Chinese descent in which at least four individuals in two successive generations have displayed clinical evidence of ALS. The pattern of inheritance of the disease trait suggests autosomal dominance. A novel mutation (A to T) in exon 5 of the SOD1 gene, which is predicted to result in the replacement of Glu by Val at codon 133 (E133V), was identified in the proband. We found no other abnormality in the PCR products of other exons. To determine whether the E133V mutation could be a polymorphism in a Chinese population or not, 50 control subjects were tested by the same method but no abnormality was found.

Discussion: In the present study, we have shown that a Chinese kindred with ALS is associated with a missense mutation in exon 5 of the SOD1 gene. The region affected by the E133V mutation is VII active site loop, which has

also been affected by more than 10 other mutations (1,2). The clinical phenotype within members of this family is relatively variable.

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P22 SOD1 GENE MUTATIONS IN ITALIAN PATIENTS WITH SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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Background: The SOD1 gene, encoding Cu/Zn superoxide dismutase, is mutated in 20% of familial amyotrophic lateral sclerosis (FALS) and about 5% of sporadic (SALS) cases. More than 100 SOD1 gene mutations have been identified.

Objectives: Analysis of SOD1 mutations in Italian ALS patients.

Methods: Mutations were searched by DHPLC and direct sequencing of the 5 exons, exon/intron boundaries and 3'UTR in 44 patients (29 male and 15 female: mean age 54.3 $\pm$ 14.5 years) consecutively referring to our ALS centre from all Italian regions. Written informed consent was obtained from all patients.

Results: We found SOD1 mutations in 3 out of 41 (7.3%) putative SALS patients and no mutations in the 3 FALS cases. In a 50-year-old female we detected the N65S mutation. Disease onset occurred about 10 years ago with a dropped foot. At the latest visit she showed a prevalent involvement of the lower motor neuron in the lower limb with a mild functional impairment (ALS-FRS = 34). Upper motor neuron involvement was expressed only by an abnormal plantar reflex. A new heterozygous change AAG to TAG, introducing a stop codon at position 136 in exon 5, was found in a 44-year-old male. The onset of the disease was characterized by a rapid progressive wasting of muscular strength with hyperreflexia in the left lower limb one year ago. At the time of our visit the patient showed upper and lower motor neuron involvement only at the spinal level with mild functional impairment (ALS-FRS = 37). The third mutation, A95T in exon 4, was identified in a 64-year-old female . Onset of the disease occurred about 20 years ago with a dropped foot. At the present time she showed a severe tetraparesis with involvement of both types of motor neurons.

Conclusions: Of the three detected mutations, N65S has been described previously in three cases, one of which was familial. They showed a slow progression of the disease with prevalent lower motor neuron involvement as in our case. The patient with the K136X mutation showed a rapid progression of the disease, in agreement with previously reported familial cases carrying truncating mutations in exon 5 (L126X and G141X). The A95T mutation, detected in a very slowly progressive patient, was previously identified in one Italian patient with juvenile onset and slow progression of the disease, but also in his unaffected relatives suggesting low penetrance of the mutation. The similarity of the clinical pictures between previously described FALS and our SALS cases links these mutations to specific clinical forms and strengthens the need to extend genetic analysis also to apparently sporadic cases.

P23 THE 'D90A' STILL AN ENIGMA AMONG SOD1 GENE MUTATIONS: REPORT OF THREE ITALIAN CASES

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Background: Of all the SOD1 gene mutations described, uniquely the D90A mutation has been identified in recessive, dominant and apparently sporadic cases. All the homozygous patients showed a typical uniform diphasic phenotype with an insidious onset. The mean age at onset is 44 years and the median survival time is 14 years. A few sporadic and familial ALS patients heterozygous for the D90A mutation have also been described, characterized by more variable phenotype and course.

Objectives: To describe atypical clinical features of two D90A heterozygous subjects belonging to unrelated families and one apparently sporadic D90A heterozygous ALS patient. Also, to evaluate a possible common haplotype of these subjects.

Methods: Mutation analysis of the SOD1 gene was carried out by single strand conformational polymorphism (SSCP) analysis followed by direct sequence of sample with abnormal migration patterns. A haplotype study was performed using eight polymorphic microsatellite markers flanking the SOD1 locus. Microsatellites were amplified by fluorescent polymerase chain reaction (PCR) primers and analysed with an automated sequencer.

Results:
Case 1. A 42-year-old male showed clinical and electrophysiological findings inconsistent with typical motor neuron disease and suggestive of a multiple sensory-motor peripheral neuropathy. His maternal grandfather died at 63 years of age with ALS. Segregation analysis of the

mutation in the family showed three healthy heterozygous relatives (45, 76 and 78 years old).

Case 2. A female with paternal history of ALS showed a bulbar onset of the disease at age 71 years and died three years later. No SOD1 mutation was found in the patient. Intriguingly, her 65-year-old healthy brother is heterozygous for the mutation.

Case 3. A 58-year-old male, heterozygous for the D90A mutation, presented progressive signs of motor neuron disease in three limbs with onset two years before. His family history was negative.

A common haplotype was found in our pedigrees and in the sporadic patient.

Discussion: These cases provide further evidence that the D90A mutation still has an unclear pathogenetic role in the disease and represents an unresolved challenge among all SOD1 mutations. Indeed, in the first pedigree a dominant inheritance with incomplete penetrance could be hypothesized. Alternatively the patient could suffer from a coincidental undiagnosed disease of peripheral nerves and belong to a recessive pedigree. In the second pedigree the mutation was found in a healthy brother but not in the patient affected by typical ALS. The third patient represents the first Italian apparently sporadic ALS case with heterozygous D90A mutation.

P24 A PHENOTYPIC-GENOTYPIC STUDY OF AMYOTROPHIC LATERAL SCLEROSIS ITALIAN FAMILIES WITH THE G41S SOD1 GENE MUTATION

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Background: More than 100 different mutations in the superoxide dismutase gene (SOD1) have been found worldwide in patients with amyotrophic lateral sclerosis (ALS), some occurring as recurrent mutations or as founder mutations. G41S is the predominant SOD1 gene mutation identified so far in ALS Italian families, together with the L84F. Occasionally, specific mutations are associated with a particular phenotype.

Objectives: To evaluate a possible genotype-phenotype correlation in six patients belonging to four unrelated familial ALS (FALS) pedigrees originating from north-west Tuscany in central Italy, carrying the G41S mutation and to investigate for a founder effect for these four families.

Methods: Diagnosis was made according to the El Escorial criteria. Genomic DNA was extracted following standard procedures. Mutation analysis of SOD1 gene was carried out by single strand conformational polymorphism (SSCP) analysis followed by direct sequence of samples with abnormal migration patterns. A haplotype study was performed using eight polymorphic microsatellite markers flanking the SOD1 locus. Microsatellites were amplified by fluorescent polymerase chain reaction (PCR) primers and analysed with an automated sequencer.

Results: Clinical phenotype was characterized by early upper motor neuron (UMN) and lower motor neuron (LMN) involvement. This occurred in lower limbs in five out of six patients and in upper limb in one out of six patients, with rapid spread to other limbs, appearance of bulbar signs within 1 year and death a few months later. In two of six patients, atypical signs (sexual, behavioural and urinary disturbances) were also found. Mean age at onset was 47.3 years and mean duration of disease, for four deceased patients, was 12 months. The G41S mutation has been previously reported in four patients from one US family presenting with a similar rapidly progressive course, but not further clinically described. All the six FALS patients showed a common haplotype covering 1.05 Mb genomic region surrounding the SOD1 gene.

Discussion and conclusions: The clinical phenotype in all patients was quite uniform and characterized by spinal onset and a very short survival. All the six FALS patients shared the same haplotype, therefore suggesting a founder effect origin for the G41S mutation. In view of this result, we propose that all the patients derive from a common ancestor.

P25 AGE OF SOD1 A4V MUTATION CAUSING ALS AND FOUNDER EFFECT

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Background: The dominant form of amyotrophic lateral sclerosis (ALS) with exclusively lower motor neuron disease was first described by Sir William Osler in 1880 in the Farr family in Vermont. In the descendents of the Farr family an alanine to valine (A4V) mutation at codon 4 in exon 1 of the SOD1 gene has been described and shown to be responsible for 50% of SOD1 mutations associated with ALS in North America. This mutation is rare in Europe. The explanation for such patterns lies in the demographic histories of populations including effects of genetic drift, migration and natural selection.

Objectives: We aimed to estimate the age of the A4V mutation and to search for a founder effect.

Methods: Ninety-six patients with confirmed A4V mutation and 96 healthy control subjects were genotyped for 14 SNPs across a 21cM region (12cM centromeric to SOD1 and 9cM telomeric). High-throughput SNP genotyping

was performed using *Taq*man assay in 384-well format on the ABI prism 7900HT sequence detection system (Applied Biosystem). Haplotype frequencies and association statistics for the polymorphisms were estimated using Haploview version 3.2; *p*-values less than 0.05 were considered statistically significant. We used a Bayesian method (using Markov chain Monte Carlo method) for multipoint linkage disequilibrium mapping incorporated in the program DMLE+ version 2.2 to estimate the age of A4V.

Results: Five SNPs located between 1cM centromeric and 2cM telomeric to SOD1 were associated with A4V ALS. A single haplotype consisting of three SNPs associated with A4V (p = 4.87e-10). Using haplotype frequency data for these three SNPs, the estimated age for A4V is 63.5 generations (~1270 years). Haplotypes for all five SNPs estimated the age to be 76.8 generations (~1536 years).

Conclusions: SOD1 A4V descended from a single founder 1200 to 1600 years ago.

P26 A NOVEL ASN86LYS MUTATION IN SOD1 CAUSES RAPIDLY PROGRESSIVE AUTOSOMAL DOMINANT AMYOTROPHIC LATERAL SCLEROSIS WITH EARLY RESPIRATORY FAILURE

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Background: Autosomal dominant familial amyotrophic lateral sclerosis (FALS) is most frequently associated with point mutations in the Cu/Zn-superoxide dismutase (SOD1) gene on chromosome 21. The corresponding changes in the structure of the SOD1 protein leads to increased oxidative stress by lipid and protein peroxidation, to increased frequency of protein aggregates and to alterations in sensitivity for proapoptotic stimuli such as increased Ca^{2+}, Fas and/or TNF sensitivity which results in degeneration of cortical and spinal motor neurons.

Objectives: A 63-year-old male presented a rapid course of a clinically definite ALS with predominantly lower motor neuron signs, severe respiratory distress but no signs of bulbar involvement. Family history revealed unclear early deaths accompanied by severe respiratory distress in the maternal line and a further case of rapid progressive respiratory failure with one sister of the index patient.

Methods: Disease progression was monitored at 3-month intervals by detailed clinical examination including FVC; functional deficits were estimated by the ALSFRS-R. The individual results were compared with pooled data from a local database. Genomic DNA was obtained from whole blood samples. The coding region and the flanking intron sequence of all five exons of the SOD1 gene were amplified by polymerase chain reaction (PCR). Single strand conformation polymorphism (SSCP) analysis was used as a screening technique and the PCR product of the conspicuous exon 4 was sequenced.

Results: Clinical follow-up showed a much more rapid disease progress in our index patient compared to the control ALS population. Genetic analysis revealed a heterozygous T to A exchange at nucleotide position 1067 in the coding region of exon 4 of the SOD1 gene resulting in an amino acid substitution of lysine for asparagine at codon position 86 (Asn86Lys).

Conclusion: A novel Asn86Lys base exchange appears to be associated with a rapid disease course and early respiratory involvement. These results expand the spectrum of known ALS associated mutations in the SOD1 gene.

P27 THE RARE EXON 4 MUTATION G93D IN THE SOD1 GENE IS ASSOCIATED WITH A SLOWLY PROGRESSIVE AND LOW PENETRANCE FORM OF ALS

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Background: The finding of rare, unknown DNA mutations in SOD1 linked to FALS is not infrequent, and a common problem for genetic counselling is poor understanding of the genotype-phenotype correlation. During the study of 12 FALS patients, we characterized a G93D (GGT > GAT) mutation in exon 4 of the SOD1 gene. This mutation has only been described in two patients, one of them of Italian origin.

Objective: To obtain a better knowledge of clinical features associated with G93D, including age at the onset of symptoms, penetrance, variation of expression and progression.

Family description: The proband is a 45-year-old female, who developed a slowly progressing muscular atrophy in lower limbs, with widespread fasciculations but without pyramidal signs. An EMG confirmed a diffuse neurogenic pattern in all limbs. Since the patient referred that her grandmother was affected by similar disturbances, an accurate family history was collected. The proband's paternal grandmother died at the age of 75 years after three years of progressive motor impairment, starting in lower limbs, without pyramidal signs. A proband's second cousin (the daughter of her grandmother's sister), born in 1938, had a diagnosis of

ALS in 1984 and is still alive. Both the proband's father and her cousin's mother, who were obligate carriers of the gene, did not develop ALS. During pre-test genetic counselling, the proband was informed about the meaning and the limits of the molecular tests and an informed consent was obtained.

Methods: Blood samples were collected and DNA purified using Perfect gDNA blood mini kit. Prior to sequencing the five coding regions of the SOD1 gene, at least 100 nt of flanking introns were analysed with DHPLC. To screen for homozygous changes, an equal quantity of normal amplicon of the five exons of the SOD1 gene was added to the PCR of the patient before heteroduplex formation. Direct sequencing of the DNA fragments showing an abnormal chromatographic profile was performed using dideoxynucleotides method with the Big Dye kit (Applied Biosystems) on an ABI Prism 3100 automated sequencer and analysed with the software 'Factura' and 'Sequence Navigator'.

Results: Sequence analysis revealed a G > A point mutation at nucleotide position 1087 in the heterozygous state in the proband, which is the same mutation found in her cousin. The mutation was absent in 121 SALS and 11 FALS cases, and in 130 healthy controls.

Discussion: The triplette GGT coding for glycine at codon 93 is a hot spot site for mutation, since it has all possible mutations in any position, giving rise to six different amino acid substitutions. For the majority of them the SOD1 activity seems quite normal and the resulting phenotype is very mild, usually slowly progressing and with an incomplete penetrance.

P28 DIFFERENT CLINICAL PHENOTYPES IN MOTOR NEURON DISEASE CAUSED BY DYNACTIN 1 (DCTN1) GENE MUTATIONS

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Different motor neuron diseases (MND) show selective vulnerability of motor neurons. A common pathomechanism is not yet known. Axonal transport seems to play an important role in the underlying mechanism. Genetically proven changes in proteins involved in axonal transport may be a basis for defined MND, e.g. amyotrophic lateral sclerosis (ALS). Based on perceptions from axonal transport in mouse model studies, it is generally accepted that mutations in the multiprotein complex dynactin are possible susceptibility factors or candidates for developing motor system degeneration. Furthermore, single MND patients have been reported with mutations in the p150 subunit of the dynactin-1-gene (DCTN1) as the underlying reason for their disease. We investigated the p150 subunit of the DCTN1 gene in 552 consecutive adult MND patients. We found different missense mutations in exons 7, 13, 15, 20, 27 and 31 and mutations in intron 2 and 28 as possible disease causing factors. None of the mutations were found in healthy controls (n = 160). In addition, we detected one polymorphism in exon 13. Analysis of phenotypes in MND patients with DCTN1 gene mutations showed a broad spectrum. We observed classical ALS patients as well as atypical MND patients with exclusive lower motor neuron affliction or uncommon clinical signs and additional abnormal brain MRI findings. In nearly all the patients, the disorders started in the upper limbs. The age at onset, individual disease progress and main clinical characteristics showed no consistent pattern in our patients.

We show that mutations in the DCTN1 gene can be associated with motor neuron degeneration, but with respect to a variable clinical phenotype, additional possible underlying pathophysiological mechanisms or other genetic or external factors have to be discussed.

P29 TRINUCLEOTIDE REPEAT EXPANSION DETECTION AT SCA2 LOCUS IN AMYOTROPHIC LATERAL SCLEROSIS PATIENTS

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Background: Spinal cerebellar ataxia 2 (SCA2) is an autosomal dominant disorder caused by CAG repeat expansions in the *SCS2* gene. The clinical phenotypes of SCA2 patients are heterogeneous. Most patients show clinical symptoms of cerebellar ataxia; other features sometimes include manifestations of Parkinsonism and motor neuron degeneration. Typically, individuals with SCA2 possess one normal SCA2 allele (14–31 CAG repeats) and one unstable expansion of the CAG repeat (>31 repeats: usual range 35–77 repeats). CAG repeat expansion at the SCA2 locus has been recognized recently as an uncommon cause of Parkinsonism both in familial and sporadic Parkinson's disease (PD). The repeat lengths in those symptomatic PD cases ranged from 33 to 43. To date, SCA2 repeat lengths have not been well characterized in ALS patients. Because there is a suggestion of motor neuron compromise with SCA2 expansions, we have investigated SCA2 repeat lengths in ALS cases.

Objective: To investigate the trinucleotide repeat range of the *SCA2* gene in familial and sporadic ALS cases.

Patients and methods: A total of 534 ALS cases (381 sporadic, 153 familial) and 418 controls were

studied using polymerase chain reaction (PCR) analysis. The amplified products of all samples were analyzed on both 3% agarose gels and 6% denaturing polyacrylamide gels.

Results: The trinucleotide repeats length of the SCA2 CAG domains in these 534 ALS patients range from 15 to 34 (predominantly 22 and 23 repeats, allele frequencies 91% and 5%, respectively). The SCA2 repeat length in 418 controls ranged from 17 to 32 (again predominantly 22 and 23 repeats, allele frequencies 93% and 4%, respectively). In 534 ALS patients, repeat lengths > 26 occurred in 29 chromosomes, including five cases showing borderline expansions (32, 32, 33, 34, 34 repeats respectively). In 418 controls, repeat lengths > 26 occurred in 5 chromosomes, with only one case showing a borderline expansion (32 repeats).

Discussion: We conclude that there is no significant difference between ALS and control DNAs with respect to the most common SCA2 repeat length. However, the numbers of repeat lengths > 26 and borderline expansions are increased nearly five-fold in ALS cases. These data suggest that SCA2 expansions warrant further investigation as factors that may confer increased susceptibility to ALS.

P30 MUTATIONAL RECOVERY OF VIRAL OPEN READING FRAMES IN SELECTED HERV-K-RELATED ENDOGENOUS RETROVIRUSES IN ALS PATIENTS

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Background: Amyotrophic lateral sclerosis (ALS) is a progressive motor neuron disease (MND) characterized by motor neuron degeneration in motor cortex, brainstem and spinal cord. The molecular pathogenesis and etiology for sporadic ALS remain unknown. Previously our group demonstrated elevated antibody reactivity to an endogenous retrovirus (HML-2/Herv-K) in patients with sporadic ALS. Other groups have found elevated reverse transcriptase activity in ALS patient plasma. The particular human endogenous retroviruses (HERVs) that are transcribed in ALS patients are unknown. It is also unclear how HERV proteins could be produced given the many inactivating mutations that are present in almost all endogenous retroviruses in the human genome.

Objective: To determine the type and quantity of endogenous retroviruses that are transcribed in the peripheral blood cells of ALS patients and controls.

Method: Whole blood was obtained from 17 ALS patients and 9 healthy controls and processed immediately into total RNA. Reverse transcriptase PCR (RT-PCR) with degenerate primers was employed to amplify the polymerase region of all HERV-K related RNAs. Subsequent amplifications used primers specific for the gag and protease region of a particular subset of Herv-K related viruses. RT-PCR products were cloned and sequenced. Sequencing data were analyzed using Vector NTI software.

Results: Greater than 95% of the RNA produced by HERVs in ALS patient or control blood samples was derived from five integrated proviruses. Sporadic ALS patients had a significantly increased nucleotide mutation rate in HERV RNAs homologous to one of these five proviruses (located in human chromosome 8p) relative to healthy controls. Further analysis of RNAs derived from the 8p and related loci found that 24% of the 17 sporadic ALS patients had mutations at up to four different nucleotides in the protease gene that corrected a premature termination of the full protease open reading frame (ORF). In contrast, similar mutations were not detected in any of the nine healthy controls.

Conclusions: Individuals with sporadic ALS exhibit an elevated rate of mutation in RNAs homologous to a particular integrated HERV in chromosome 8p. In 24% of the patients, these mutations would allow for the translation of full length viral gene products that could not be transcribed from the 8p HERV sequence integrated in the human genome. These results help explain appearance of an immune response to HERV proteins in ALS patients. The elevated mutation rate of HERV 8p related RNAs is consistent with reverse-transcriptase mediated replication of this HERV in some sporadic ALS patients.

P31 PESTICIDES, PARAOXONASE AND SPORADIC ALS

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Background: Sporadic ALS (SALS) appears to have an increased incidence in Gulf War veterans (1), in people exposed to agricultural chemicals or living in rural areas, and in airline pilots (2). All of these are exposed to organophosphates. Organophosphates are hydrolyzed by the enzyme paraoxonase 1 (PON1). Certain polymorphisms in the promoter and exons of the PON1 gene can increase or decrease the activity and expression of PON1 (3). Any impairment of PON1 could allow organophosphates to attack motor neurons.

Objectives: To investigate the interactions between PON1 polymorphic genotypes and pesticide exposure in SALS.

Methods: PON1 promoter SNPs (−909c > g, −832g > a, −162g > a and −108c > t) and coding SNPs (L55M and Q192R) were studied in 143 SALS cases and 143 controls matched for age, sex and ethnicity. Alleles, genotypes and haplotypes were compared between

SALS cases and controls. Logistic regression was used to investigate gene-environment interactions.

Results: The frequency distribution of the −108c > t allele was different in cases and controls ($p = 0.03$). All four promoter genotypes were associated with SALS. Of interest, the high-expression variants −108c > t CC (OR = 0.12, CI 0.03−0.46, $p = 0.01$) and −162g > a AA (OR = 0.04, CI 0.01−0.29, $p = 0.01$) were negatively correlated with SALS. The low-expression haplotype at these SNPs was weakly associated with SALS. The coding L55M and Q192R genotypes and haplotypes were not associated with SALS. An analysis of gene-environment interaction showed that people with PON1 susceptibility alleles at each of the six SNPs, who were also exposed to pesticides, were even more likely to have SALS than if they had the susceptibility alleles alone (ORs between 1.81 and 2.39).

Discussion and conclusion: Promoter genotypes that control the expression of the PON1 enzyme were associated with SALS. In particular, the −108c > t polymorphism, which has a large effect on PON1 expression, may be important. The interaction of the PON1 alleles and pesticide exposure was only slightly increased compared to allele susceptibilities alone, implying that genetic susceptibility is the major component underlying this association.

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P32 GENOTYPE AND EXTREME EXERCISE – A LETHAL COMBINATION IN ALS?

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Background: Athleticism has been identified as a possible risk factor in ALS. Large scale case controlled epidemiological studies have failed to confirm this observation. Confounders in such studies have included overmatching of controls, and failure to stratify ALS patients by gender, age of onset, and site of onset. Hypoxia-inducible factors including vascular endothelial growth factor (VEGF) and angiogenin have been implicated in the pathogenesis of ALS. 'At-risk' alleles have been identified in VEGF in some populations.

Objective: To conduct a detailed case controlled study of lifetime metabolic activity in ALS patients and controls, and to genotype the ALS population with respect to 'at-risk' alleles in VEGF and angiogenin.

Methods: Over 100 ALS and matched controls completed a detailed questionnaire about their lifetime history of exercise and metabolic expenditure. The disease and control populations were divided into cohorts comprising those with low, medium, high (>1 standard deviation from the mean) and extreme (>2 standard deviations from the mean) histories of lifetime metabolic expenditure. Disease and control populations were genotyped for 'at-risk' angiogenin and VEGF haplotypes.

Results: There was a disproportionately high number of males with spinal onset ALS who had a history of high or extreme metabolic expenditure. The −2578C homozygous VEGF genotype was identified in 70% of individuals with high or extreme energy expenditure (EEE), compared to 29% of ALS patients with medium energy expenditure, and 22.6% of controls. This genotype has been previously reported to be associated with increased risk of early age of onset. The −2578C, −1154G and −634C haplotype was identified in 70% of patients with a history of EEE, compared to 22% of patients with medium expenditure, and 18% of controls. Sixty percent of patients who were −2578C homozygous also carried the angiogenin 'at risk' G allele of rs11701 SNP.

Conclusions: Exercise may combine as an environmental susceptibility in ALS patients with VEGF and angiogenin 'at risk' haplotypes.

P33 ANALYSIS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) HAPLOTYPES AND RISK FOR ALS IN NORTH AMERICAN, IRISH AND SCOTTISH POPULATIONS

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Background: VEGF has been implicated in the pathogenesis of ALS. In a meta-analysis of over 900 ALS patients and more than 1000 controls from northern Europe, individuals homozygous for the VEGF haplotypes (−2578A/−1154A/−634G/ or −2578A/−1154G/−634G/) had a 1.8 times greater risk for the development of ALS. These at-risk haplotypes were associated with reduced VEGF expression and lowered serum VEGF levels.

Objectives: We sought to examine these at-risk haplotypes in the North American, Irish and Scottish populations.

Methods: We examined the VEGF haplotypes in a total of 466 sporadic ALS (SALS), 77 familial ALS (FALS) (no SOD1 mutations) and 408 matched control samples.

Results: Analysis of the North American population identified an increase in frequency of the −2578C, −1154G and −634C alleles in SALS and FALS compared to controls ($p > 0.024$). However, there was no significant difference in the distribution of the previously identified at-risk VEGF haplotypes in SALS/FALS compared to controls ($p > 0.05$). Analysis of the VEGF haplotype in the Irish population showed no significant difference between SALS individuals and controls ($p > 0.36$). Further analysis of the Irish population demonstrates a trend towards increased frequency of the −2578C, −1154G and −634C alleles in specific subgroups. However, combined analysis of the North American, Irish and Scottish populations showed no significant difference in the at-risk VEGF haplotype when SALS were compared to controls ($p > 0.3$).

Conclusions: There is increasing evidence strongly suggesting a biological role for VEGF as a modifier of motor neuron degeneration. However, this study does not replicate the haplotype association identified in a large cohort of Northern Europeans. Other haplotypes may be important in conferring risk in subpopulations of ALS.

P34 ASSOCIATION BETWEEN HFE MUTATIONS AND ALS

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Background: Oxidative stress is considered to play a key role in the process leading to motor neuron degeneration. Iron is a very potent pro-oxidant and its metabolism can be disrupted due to mutations in the *HFE* gene. Recent studies suggested HFE mutations to be a risk factor for various other diseases, including Alzheimer's disease. This prompted investigators to study the relationship between HFE mutations and the development of ALS. One study showed that an H63D mutation was associated with an increased risk of developing ALS.

Objectives: To investigate the possible association between the presence of a C282Y or an H63D HFE mutation and the development of ALS. Furthermore, to determine a possible effect of HFE mutations on disease characteristics.

Methods: Two hundred and eighty-nine ALS patients were randomly selected. Genotyping was performed for both the C282Y and the H63D mutations. Controls were taken from two population based studies carried out among individuals living in the same region. A random sample of 5886 individuals were genotyped for both HFE mutations. Logistic regression analysis adjusted for known risk factors was performed.

Results: We found that homozygous mutations at H63D were independently associated with an increased risk of developing ALS (OR = 2.16, $p = 0.02$). The remainder of genotypes were not associated with ALS. The presence of the C282Y or H63D mutations did not significantly affect site of first weakness or survival. However, carrying an H63D mutation was associated with a higher age at onset (OR = 0.73, $p = 0.033$).

Discussion: One study reported a significant association between HFE mutations and the development of ALS. However, this was based primarily on H63D heterozygotes. In our study of larger sample size, only H63D homozygosity was significantly associated with an increased risk of ALS. The difference in genetic background of the population and the fact that we used a large cohort of population-based controls could account for these differences. These findings suggest a role of HFE mutations in development of ALS, although the underlying mechanism is still uncertain. A possible association with iron overload is the subject of a future study.

P35 SOD1 DOWN-REGULATION IN ALS PATIENTS CARRYING AN HFE MUTATION

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Background: *Hfe* mutations are associated with an increased risk of iron loading in the body, which in turn is associated with an increased risk of oxidative stress and altered inflammatory reactions. We have reported that 31% of sporadic ALS patients carry an *Hfe* mutation compared to only 14% of non-ALS patients with some type of neuromuscular complaint ($p<0.005$). The worldwide prevalence of *Hfe* mutations in the general population is reportedly 10–12% which is similar to that reported for the non-ALS group. The prevalence of this mutation is the second highest genetic mutation ever identified within the ALS population. Of the two most common types of *Hfe* mutation, the H63D mutation is the specific *Hfe* mutation associated with ALS.

Objectives: Our hypothesis is that *Hfe* mutations establish a permissive cellular environment that will enable genetic factors that promote the induction of ALS.

Methods: To test this hypothesis, we developed two stable cell lines from a human neuronal cell line (SH-SY5Y) that carry either the H63D mutation or C282Y mutation of the *Hfe* gene. We determined SOD1 expression by Western blotting and mitochondria membrane potential (MMP) using JC-1 staining. We also examined SOD1 expression in human muscle biopsy samples obtained during standard diagnostic procedures for ALS. Only those individuals with the H63D mutation have been examined because this was the most prevalent mutation in the ALS samples.

Results: The *Hfe* mutant cell lines had a decrease in SOD1 expression and in particular the level of SOD1 in the H63D cell line is lower than the cell line carrying the C282Y mutation or the wild-type control cell line. In addition, the greatest decrease in MMP occurred in the H63D mutant cell line. Based on the cellular analysis, we examined SOD1 expression in the muscle tissue of the ALS patients and found those individuals with ALS that carry the H63D mutation ($n = 14$) have one-third less SOD1 expression ($p < 0.05$) in muscle tissue compared to ALS patients with wild type *Hfe* ($n = 18$). The SOD1 expression in the muscle biopsies was nearly identical to the results for the *Hfe* transfected cells.

Conclusions: These data suggest that those individuals with ALS who carry an *Hfe* mutation have an increase in oxidative stress compared to individuals with ALS who do not have this mutation. The data also suggest the cell lines provide a novel and reliable system for identifying the mechanism by which the H63D mutation affects ALS. Studies are ongoing to identify additional biomarkers that distinguish between ALS patients with and without the *Hfe* mutation. This information is important for understanding how *Hfe* mutations are a risk factor for ALS and for developing intervention strategies.

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P36 DETOXIFICATION GENE POLYMORPHISMS AND SUSCEPTIBILITY TO SPORADIC MND IN A RUSSIAN POPULATION

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Background: A significant pathogenic factor of motor neuron disease (MND) may be oxygen free radicals, and detoxification is an important metabolic process in which free radicals might be formed and subsequently transformed into non-toxic products.

Objectives: We studied polymorphisms in the genes of detoxification systems in ALS patients and controls.

Materials and methods: We studied a 96bp insertion polymorphism of the cytochrome *2E1* gene (CYP2E1*1D), CYP2D6*4 polymorphism of the cytochrome *2D6* gene, deletion polymorphism of glutathione-S-transferases T1, M1 and P (GSTM1, GSTT1 and GSTP), and slow-rapid acetylation polymorphism of N-acetyltransferase type 2 gene (*NAT2*) in 75 patients with sporadic MND within the age range 28–72 years and 105 randomly sampled controls from Moscow. Analysis was performed using PCR and autoradiography of PCR products, with lengths corresponding to allelic variants of the named genes. The diagnosis of MND was made according to revised El Escorial criteria (1998); rapid (>10 degrees per six months), moderate (5–10 per six months) and slow (<5 per six months) progression rates were designated according to loss of Norris ALS Score degrees.

Results: A considerably increased frequency of CYP2E1*1D was observed in MND patients (14% versus 2.5% in controls; $p < 0.001$). Analysis of genotype distributions did not identify the CYP2E1*1D/CYP2E1*1D genotype in the control group. Comparative analysis of genotype distributions showed statistically significant differences between the patients and controls in our population ($p = 0.0018$). The GSTT1(0/0)/GSTM1(0/0) and GSTT1(+)/GSTM1(0/0) genotype combinations prevailed among the controls ($p = 0.033$) and GSTT1(0/0)/GSTM1(+) and GSTT1(+)/GSTM1(+) combinations prevailed among MND patients. In contrast, the analysis for CYP2D6, GSTT1, GSTP1 and NAT2 gene polymorphisms has revealed no differences in distribution of different genotypes and alleles between patients and controls. There were no associations found between polymorphic variants and clinical features of MND (ALS/PBP, progression rates).

Conclusion: These data suggest that the CYP2E1*1D allele is associated with sporadic MND and is involved in the pathogenesis of sporadic MND in Russian patients, being possibly associated with production of higher levels of toxic metabolites of xenobiotics of Phase I of detoxification.

P37 GENETIC RISK FACTORS FOR SPORADIC AMYOTROPHIC LATERAL SCLEROSIS (SALS)

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Background: ALS is the neurodegenerative disorder of still unknown aetiology. The mutations of SOD1 have been shown as a risk factor of familial ALS (FALS). Polymorphisms of different genes involved in the processes associated with possible mechanisms of motor neuron degeneration due to ALS, may play a role as risk factors of ALS.

Objective: To study the polymorphisms of the genes that are potentially involved in the pathogenesis of SALS, i.e. inflammation (interleukin-1 −511 C/T, interleukin-6 −174 G/C polymorphisms, SERPINA3 A/T polymorphism); oxidative stress (paraoxonases (PON) Gln192Arg PON1 Leu55Met and PON2 Cys311Ser and metalloproteinase-9 C/T polymorphisms).

Material and methods: We included 167 patients with definite or probable diagnosis of SALS and 452 healthy controls matched for age and sex. The diagnosis of ALS was established according to El Escorial criteria (1994). The FALS cases were excluded on the basis of the positive

family history of ALS. The polymorphisms were studied using PCR technique and restricted enzyme digestion.

Results: The study showed that among all studied polymorphisms the distribution of the following polymorphisms differs between ALS cases and controls: PON1 Gln192Arg polymorphism [cases (n = 166): Gln/Gln – 72, 47.6%; Gln/Arg – 67, 40.4%; Arg/Arg – 20, 12.0% vs. controls (n = 440): Gln/Gln – 242, 55%; Gln/Arg – 167, 38%; Arg/Arg – 31, 7%, p = 0.032] , PON2 Cys311Ser polymorphism [cases (n = 166): Cys/Cys – 17, 10.2%; Cys/Ser – 71, 42.8%, Ser/Ser – 78, 47%, controls (n = 397): Cys/Cys – 28, 7.1%, Cys/Ser – 133, 33.5%, Ser/Ser –236, 59.4%, p = 0.006], and SERPINA3 A/T polymorphism only in bulbar onset SALS [cases: (n = 43): CC –7, 16.3%, CT – 19, 44.2%, TT – 17, 39.5%, genotype distribution in controls (n = 404): CC – 106, 26.2%, CT – 193, 47.8%, TT – 105, 26.0%, p = 0.05].

Conclusion: Our findings suggest that among studied polymorphisms only those related to oxidative stress may be involved in the risk of SALS.

P38 METHYLENETETRAHYDROFOLATE REDUCTASE GENE POLYMORPHISMS IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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Background: The N5, N10-methylenetetrahydrofolate reductase catalyses the remethylation of homocysteine to methionine and the biosynthesis of nucleotides. Two polymorphisms, C677T and A1298C of the *MTHFR* gene have been identified, and associated with cardiovascular diseases, neurovascular, neurodegenerative diseases, psychiatric disorders and cancer. Amyotrophic lateral sclerosis (ALS) is a chronic progressive devastating disease of the central nervous system, characterized by the death of upper and lower motor neurons.

Objectives: The aim of the study was to determine whether the MTHFR C677T and A1298C polymorphisms were genetic risk factors for ALS in Turkey.

Results: A total of 52 ALS patients and 278 controls were genotyped for the MTHFR C677T and A1298C polymorphisms. The 677T allele frequency of the *MTHFR* gene was 30.77% in the ALS patients and 35.97 in the healthy controls. The 1298C allele frequency of the *MTHFR* gene was 37.5% in the ALS cases and 35.43% in the controls. The MTHFR C677T genotype showed a 1.9-fold increase risk for ALS (OR = 1.916; 95% CI 1.033–3.554) χ^2 = 4.351; df = 1; p = 0.037). Likewise the C677T/A1298C compound genotype showed a 2.4-fold increase risk for ALS (OR = 2.434; 95%CI 0.996−5.952; χ^2 = 4.013; df = 1; p = 0.045).

Discussion: To our knowledge, this is the first evidence showing an association between the MTHFR polymorphisms and ALS. Although we only analysed 52 sporadic ALS patients and 278 healthy controls, the association is only significant in the genotypes C677T and C677T/A1298C. However, there was no allele association. To clarify the data, more ALS patients should be studied in the future.

P39 MUTATION SCREENING OF THE *VAPB* GENE IN SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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Background: Amyotrophic lateral sclerosis (ALS) is a heterogeneous, progressive, degenerative disease characterized by loss of motor neurons in the spinal cord, brain stem and motor cortex. To date, five genes have been found to be associated with familial ALS (FALS). A mutation in exon 2 (166, C→T) of vesicle-trafficking protein *VAPB* gene has been found in seven families with diagnoses ranging from typical ALS to mild spinal muscular atrophy (SMA) (1). Their common clinical presentation is an autosomal dominant slowly progressive disorder characterized by fasciculations, cramps and postural tremors. Historical data indicate that the seven families have a common Portuguese ancestor and probably belong to a single large family.

Objective: To screen the *VAPB* gene for mutations in sporadic ALS (SALS).

Methods: DNA from 90 sporadic cases and one familial case was screened for mutations in *VAPB* gene. Primers were designed by primer 3 software to amplify all six exons plus the 5' and 3' untranslated regions. The products were analyzed by dideoxy termination sequencing on a Beckman coulter 8000. The sequences were aligned by Sequencher V4.2 and analyzed by eye. Each variant identified was also analyzed among 100 control subjects.

Results: We detected the known mutation, C to T at nucleotide 166 (Pro56Ser) in the familial ALS case which is from the same geographical region as the reported families. The same mutation was not found in SALS cases.

Discussion: Mutations in the *VAPB* gene are not a common cause of sporadic ALS.

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P40 POLIO VIRUS RECEPTOR-RELATED TYPE 2 (*PVRL2*) GENE IS ASSOCIATED WITH SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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Background: Amyotrophic lateral sclerosis (ALS) is a rapidly progressive, age-dependent, neurodegenerative disorder of motor neurons with both sporadic and familial forms. The cause of most types of ALS is unknown and the disease is untreatable. We have recently identified chromosome 19q13 as a candidate susceptibility locus in sporadic ALS (SALS).

Objective: To identify susceptibility polymorphisms in candidate genes on chromosome 19q13 for sporadic ALS.

Methods: This association study of a North American Caucasian population included 14 polymorphisms spanning PVRL2 and TOMM40 in 128 parent-child triads (trios) and 192 SALS patients, age and gender matched with 192 control subjects. PVRL2 functions as a herpes virus receptor and TOMM40 is an outer membrane protein of the mitochondria that helps import nuclear coded proteins into the mitochondria. High-throughput SNP genotyping was performed using *Taq*man assay in a 384-well format on the ABI prism 7900 sequence detection system (Applied Biosystem). Haplotype frequencies and association statistics for the polymorphisms were estimated using Haploview version 3.2. *P*-values less than 0.05 were considered statistically significant.

Results: The polymorphism rs3745150, located in the intergenic region between PVRL2 and TOMM40, demonstrated an association with SALS ($\chi^2 = 5.172$, 1df, $p = 0.023$) in the case-control model. The association was borderline in the trio sample ($\chi^2 = 3.279$, 1df, $p > 0.05$). However, the polymorphism rs2927466, located in intron 2 of PVRL2, was over-transmitted to affected offspring ($\chi^2 = 4.149$, 1df, $p = 0.0417$). None of the TOMM40 polymorphisms showed an association in either the case-control or the trio model.

Conclusion: PVRL2 polymorphisms associated with SALS in the case-control and trio groups. This study lends support to the hypothesis of neuronal vulnerability associated with polymorphisms in molecules utilized by viruses for their entry.

P41 A PHENOTYPIC-GENETIC STUDY OF NINE POLISH MEN WITH SPINAL BULBAR MUSCULAR ATROPHY (SBMA)

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Background: SBMA (Kennedy's disease) is a rare, adult form of X-linked recessive neurodegenerative disorder caused by the expansion of a polymorphic trinucleotide CAG-repeat sequence in the first exon of the androgen receptor (*AR*) gene. The CAG repeat within the *AR* gene is polymorphic in healthy individuals, ranging in size from 5 to 33 repeats. In SBMA patients, however, the CAG repeat ranges in size from 40 to 62 repeats. Classically, patients are presented with proximal spinal and bulbar weakness and atrophy, generalized fasciculations, sensory involvement and slow progression.

Methods: We examined nine males (from seven families) with clinical phenotype of SBMA and three female carriers from two families. The patients underwent the standard neurological examination and neurophysiological studies. Serum levels of creatine kinase (CK) and hormones (testosterone, LH, FSH, PRL) were measured. DNA was extracted from peripheral blood according to standard procedures for banking. The detection of a pathologically expanded CAG sequence in the *AR* gene was performed by polymerase chain reaction (PCR) techniques.

Results: Male patients (mean age: 45.1 $\pm$ 13.1 years; 20–70 years) presented the history of progressing distal(8/9) or proximal (1/9) limb and facial muscular weakness with orofacial fasciculations (9/9), amyotrophy of different distributions, nasal voice (9/9), dysphagia (9/9), hand tremor (5/9), distal peripheral sensory disturbances (2/9) and gynecomastia (8/9) as well as impotence (8/9). The 'quivering chin' phenomenon occurred in 7/ 9 men. There was no evidence of upper motor neuron involvement. One of the examined female carriers presented with a 30-year history of fasciculations and minimal distal weakness and cramps in the legs, while the remaining two were asymptomatic. DNA analysis revealed expanded size of CAG repeats in Xq11-12 in the *AR* gene in all males (range 26–52 CAG repetitions) and in females (range 46–48 CAG repetitions). Neurophysiological studies showed signs of chronic denervation in all males studied. There was no correlation between CAG repetition size and the age of disease onset and duration. However, we found significant reverse correlation between CAG repetition length and level of testosterone and prolactin.

Conclusions: The correlation between the repeat length and the severity and earlier onset of the disease has been often described in the literature; however, we did not find this correlation in the patients studied. Our patients are presented with less common phenotype of SBMA with distal muscle weakness.

P42 SPG3A MUTATION SCREEN IN A COLLECTION OF NORTH AMERICAN HEREDITARY SPASTIC PARAPLEGIA CASES

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Background: The hereditary spastic paraplegias (HSPs) are a clinically and genetically heterogeneous group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness often associated with bladder disturbance (1). Identification of 11 HSP genes has shown that several pathophysiological pathways are involved in this disease including impairment of axonal transport, a common link with other neurodegenerative diseases (2). The gene mutated at the SPG3A locus encodes atlastin, a protein localized to the golgi apparatus (3). To date over 20 mutations have been identified in individuals with early onset HSP. These include missense changes and a frame shift mutation that leads to a slightly truncated protein.

Objectives: To determine the frequency of SPG3A mutations in a collection of 70 North American HSP cases and to characterize the nature of these mutations.

Methods: Primers were designed to amplify the 14 exons and intronic flanking sequence. The amplicons were analyzed by dHPLC WAVE and the variants were sequenced. The control samples were tested by direct sequencing. Standard Western blot and RT PCR analysis were performed on lymphoblast cell line extracts.

Results: We identified one segregating variant in a French Canadian family that was absent in 80 control samples. This variant leads to an in frame deletion of N436, a predicted glycosylation site (3). Western blot analysis of the patient sample did not show an altered migration pattern of atlastin, but revealed a reduction in protein quantity. RT PCR analysis suggests that this stems from a reduced atlastin mRNA level in lymphoblasts.

Conclusion: Our data suggest that the N436 site does not affect glycosylation and that this mutation causes HSP by haplo-insufficiency. Additional quantitative studies and development of a zebra fish knockdown model are underway to better understand the role of atlastin in maintenance of healthy corticospinal tract neurons.

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P43 ACQUIRED NUCLEIC ACID CHANGES MAY TRIGGER SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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This presentation brings together evidence to support the hypothesis that acquired nucleic acid changes are the proximate causes, 'triggers', or 'initiators' of sporadic amyotrophic lateral sclerosis (ALS). Clinical features that support this hypothesis include: focal onset and spread, and the individualized rate of progression. Clues from the epidemiology of sporadic ALS include the increase in its age-specific incidence with age, suggesting accrual of time-dependent changes, increased frequency of monoclonal gammopathy of uncertain significance in patients with ALS, suggesting shared risk factors, and the emergence of smoking, having a known carcinogen, as its first 'more likely than not' exogenous risk factor. The identification of any exogenous risk factor suggests that a large proportion of sporadic cases have a triggering mechanism susceptible to that factor. Ingestion of the products of *Cycad circinalis* has been hypothesized to be implicated in causing western Pacific ALS. *Cycad* contains both neurotoxic factors and carcinogens. The dissimilarity between western Pacific ALS and neurotoxic diseases suggests greater likelihood that the effects of DNA alkylation are its proximate cause. Evidence in support of a hypothesis does not constitute proof of the hypothesis, but sets the stage for its further consideration (1).

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P44 INCIDENCE OF ALS IN BELGRADE, 1992–2004 PRELIMINARY DATA

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Background: Most of the epidemiological studies of ALS have reported an increased incidence in the last three decades.

Objectives: To estimate the incidence of ALS in the population of Belgrade during the period of 1992–2004.

Methods: ALS cases were collected by analysing hospital in- and out-patient registers at the Institute

of Neurology, which is the national referral neurological centre, and in departments of neurology in an additional three clinical centres in Belgrade. The El Escorial diagnostic criteria for ALS were applied to all cases enrolled in the register. Each patient was regularly followed up during the disease. The incidence rates were calculated by standard procedures and the calculation of confidence intervals was based on Poisson's frequency distribution for rare events.

Results: In the period l992–2004, 244 (150 male and 94 female) patients with ALS were identified in The District of Belgrade. The mean age of onset was 59.5 $\pm$ 11.2 (range 25–87) years. The overall average annual incidence rate of ALS was 1.2/100,000 (95% CI 0.9–1.4); 1.5/100,000 (95% CI 1.1–2.0) for males, and 0.9/100,000 (95% CI 0.7–1.1) for females. The highest age-specific incidence rate was registered in the age group 60–64 (3.5/100,000). The incidence rate for spinal onset of ALS was 0.85/100,000, and for bulbar onset 0.25/100,000. During the observed period the incidence rate of ALS in Belgrade showed a statistically significant increasing tendency ($y = 0.783 + 0.058x$, $p = 0.047$).

Conclusion: In comparison to our previous data (1) the results suggest a significant increase in the incidence of ALS in Belgrade.

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P45 RISING PREVALENCE IN 2004 IRISH ALS POPULATION

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Background: The Irish ALS Register was established in 1993 and has full case ascertainment from 1995 onwards. During the last decade there have been rapid advances in the treatment of ALS including improved utilization of gastrostomy nutrition, the introduction of non-invasive ventilation and the availability of riluzole.

Objectives:

1) To determine the incidence and prevalence of ALS in Ireland during the period 1995–2004.
2) To examine the temporal trend of ALS in Ireland by comparing the incidence and prevalence in the three-year period 1995–1997 with the three-year period 2002–2004.

Methods: The Irish ALS Register collects information on all patients diagnosed with ALS in Ireland using multiple sources of information.

Results: The average annual incidence for the three-year period 2002–2004 was 2.1 per 100,000 person-years and 2.9 per 100,000 person-years among the population older than 15 years. This is almost identical to the incidence figures reported for 1995 –1997 (2.1 and 2.8 per 100,000 person-years). In contrast, the crude prevalence on 31 December 2004 was 7.6 per 100,000 population and 10.2 per 100,000 population over the age of 15 years. These figures were statistically significantly higher than the prevalence figures on 31 December 1996, namely 4.7 per 100,000 of the total population (z test = -1.93, $p = 0.053$), and 6.2 per 100,000 for the population older than 15 years (z test = -2.22, $p = 0.026$). The median survival of an individual diagnosed with ALS in 2004 is longer than that for a patient diagnosed in 1996.

Discussion: The prevalence rate of ALS in Ireland has risen by 65% between 1996 and 2003, but the incidence rates have remained unchanged over the same time period.

Conclusion: This finding is due to improved prognosis among Irish ALS patients and may reflect the improved availability of multi-disciplinary healthcare for this patient group.

P46 RILUZOLE AND ALS SURVIVAL: A POPULATION-BASED STUDY IN SOUTHERN ITALY

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Background: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting motor neurons, for which there is no effective cure. Riluzole is to date the only treatment that prolongs ALS survival, as evidenced by two clinical trials. However, results on the efficacy of riluzole in observational population-based studies with a longer follow-up are conflicting and, therefore, it is still unclear if the effect of the drug is limited to an early stage of the disease and to some specific subgroups of patients, such as subjects with younger age or with bulbar onset.

Objectives:

1) To evaluate the effect of riluzole on ALS survival at two years in a cohort of incident cases.
2) To examine whether bulbar ALS benefits from the medication to a greater extent.
3) To assess the efficacy of the drug in elderly patients.

Methods: Source of the study was a prospective population-based registry of ALS established in Puglia, Southern Italy, in 1997. We examined survival of 126 out

of 130 incident ALS cases that were diagnosed during the period 1998–99.

Results: Seventy-three patients (58%) were prescribed riluzole and the remaining 53 (41%) were not. Riluzole therapy increased survival rates at 12 months by approximately 10% and prolonged survival by six months (18.2 months versus 12.4; peto test; 2.78; $p = 0.09$). This beneficial effect was present among bulbar onset ALS (peto test: 4.11; p, 0.042), but not in subjects with limb onset (peto test: 0.48; $p = 0.4$). In patients aged more than 70 years riluzole treatment was associated with an eight- months longer median survival time (15.4 months versus 7.1; $p = 0.03$) and a reduction in mortality rate at 12 months by 27%, regardless of site of symptom onset. Riluzole use was an independent predictor of survival at 12 months from the diagnosis in this series in multivariate analysis with borderline significance (HR: 0.5; 95% CI 0.2–1.02; $p = 0.06$). Riluzole was effective among cases with bulbar onset ALS (HR: 0.29; 95% CI 0.08 −0.98; $p = 0.05$) and elderly patients (HR: 0.32; 95% CI 0.1−0.99; $p = 0.05$), whereas in subjects with limb onset there was no effect on survival at 12 months (HR 0.72; 95% CI 0.30−1.75). In each model riluzole did not influence survival at 24 months.

Conclusions: In this population-based series, we found that riluzole therapy improves ALS survival. The efficacy of the drug was present among bulbar onset ALS and older patients, but not in subjects with limb onset. The favourable effect of the drug was transient, as it was lost in prolonged follow-up. Our observations support the use of riluzole at an early stage of ALS in bulbar and elderly patients. The appropriate duration of riluzole treatment remains, however, to be established.

SLAP Neurologists: G Belfiore, G Benedetto, N Cacudi, A Cazzato, P Colamartino, P Di Viesti, S Epifani, F Lincesso, B Maggio, V Monitillo, A Moramarco, A Nicolaci, C Nozzoli (Brindisi), Sergio Pasca (Casarano), Rosaria Pulimeno (Gallipoli), Giuseppe Russo, V Santamato, IL Simone, G Strabella, M Terraciano, P Tota, F Valluzzi.

P47 INCREASED RISK FOR DEVELOPING ALS IN THE MILITARY POPULATION: FURTHER EVIDENCE FROM THE FRENCH POPULATION

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Background: Recent works have underlined an apparent increased risk for developing ALS for veterans from the Gulf war and then more globally in the military population. To date, the reasons for such an increased risk remain unclear. It has been supposed that it is due to particular expositions during conflicts. It is also likely that it is simply due to the profession itself and the multiple expositions encountered during the professional course.

Objectives: To determine the percentage of military personnel in an unselected ALS population and analyse the type of activity to try to define some specific exposure.

Methods: Three hundred and eleven consecutive ALS patients followed in our clinic during the last three years were interrogated for their past or present professional activity in the army. When present, the rank (officer or not) and service (air force, marine, ground, gendarme) were also collected.

Results: There were 21 patients belonging to the military population either retired or not, comprising 18 males and 3 females. Characteristics of ALS were not different from classical ALS. The observed frequency was compared to the proportion of military personnel in our country. The relative risk (RR) to develop ALS was 4. There was no influence of the rank of patients. Conversely, patients who worked in the Air Force had a significantly higher risk with a RR of 10.

Discussion: The increased risk for developing ALS in military personnel is also found in the French population. A significant predominance of subjects from the Air Force is noted in our country in contrast to other services. However, this is not a systematic survey of the military population in the whole country or a case control study. On the other hand, we interrogated 311 consecutive patients, without selection. Our region (Languedoc-Roussillon) does not have a significant number of military bases. Thus, this does not seem to represent a possible bias. We are now planning a large national survey to include a systematic questionnaire of ALS patients to identify environmental risk factors. Indeed, military personnel are likely to be exposed to a large variety of potentially neurotoxic factors or suspected risk factors for ALS such as radiation (electromagnetic or ionizing), solvents, heavy metals, vaccinations, infectious diseases, trauma, or physical activity.

Conclusion: An increased risk for developing ALS was found among 311 consecutive ALS patients followed in our MND clinic. This risk is particularly high in those personnel belonging to the Air Force. Work is in progress to confirm these results in the whole country and to identify environmental risk factors in that population.