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ORIGINAL INVESTIGATION

**Giuseppina Carrieri · Massimiliano Bonafè · Maria De Luca · Giuseppina Rose · Ottavia Varcasia
Amalia Bruni · Raffaele Maletta · Benedetta Nacmias · Sandro Sorbi · Francesco Corsonello · Emidio Feraco
Kirill F. Andreev · Anatoli I. Yashin · Claudio Franceschi · Giovanna De Benedictis**

Mitochondrial DNA haplogroups and APOE4 allele are non-independent variables in sporadic Alzheimer's disease

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Abstract Allele ε4 of the nuclear APOE gene is a leading genetic risk factor for sporadic Alzheimer's disease (AD). Moreover, an allele-specific effect of APOE isoforms on neuronal cell oxidative death is known. Because of the role of the mitochondrial genome (mtDNA) in oxidative phosphorylation and oxidative stress, an interaction between APOE polymorphism and mtDNA inherited variability in the genetic susceptibility to sporadic AD can be hypothesized. We have explored this hypothesis by analyzing mtDNA germline variants (mtDNA haplogroups) in a sample of AD patients (213 subjects) genotyped for APOE and classified as APOE ε4 carriers and non-carriers. We found that the frequency distribution of mtDNA haplogroups is different between ε4 carriers and non-carriers (P=0.018), thus showing non-random association between APOE and mtDNA polymorphisms. The same analysis, carried out in two samples of healthy subjects (179 age-matched and 210 individuals aged more than 100 years), showed independence between ε4 allele and mtDNA haplogroups. Therefore, the APOE/mtDNA interaction is restricted to AD and may affect susceptibility to the disease. In particular, some mtDNA haplogroups (K and U) seem to neutralize the harmful effect of the APOE ε4 allele, lowering the ε4 odds ratio from statistically significant to non-significant values.

G. Carrieri · M. De Luca · G. Rose · O. Varcasia
G. De Benedictis (✉)
Department of Cell Biology, University of Calabria,
87030 Rende Italy
e-mail: g.debenedictis@unical.it,
Tel.: +39-0984-492931, Fax: +39-0984-492911

M. Bonafè · C. Franceschi
Department of Experimental Pathology, University of Bologna,
Bologna, Italy

A. Bruni · R. Maletta
Regional Neurogenetics Center, Lametia, Italy

B. Nacmias · S. Sorbi
Department of Neurological Sciences, University of Florence,
Florence, Italy

F. Corsonello · E. Feraco · C. Franceschi
Italian National Research Center on Aging (INRCA).
Ancona and Cosenza, Italy

K. F. Andreev · A. I. Yashin
Max Planck Institute for Demographic Research,
Rostock, Germany

Introduction

Several lines of evidence suggest that abnormalities in oxidative metabolism and bioenergetic defects, specifically in oxidative phosphorylation (OXPHOS) enzymes, play a role in Alzheimer's disease (AD; Blass and Gibson 1991; Shoffner 1997; Gibson et al. 1998). Oxidative metabolism occurs in mitochondria, which have their own DNA. Human mitochondrial DNA (mtDNA) is a circular closed molecule of 16,569 nucleotides that codes for 13 polypeptides essential for OXPHOS enzymes plus rRNAs and tRNAs necessary for mitochondrial protein synthesis (Anderson et al. 1981; Wallace 1994). MtDNA has unique genetic features. It is independently replicated, transcribed, and translated, is maternally inherited, does not recombine, and undergoes replicative segregation during both mitosis and meiosis. The notable production of oxygen-free radicals that occurs within the mitochondrion causes mtDNA damage (somatic mutations) that accumulates in post-mitotic tissues, thus causing a heteroplasmic condition (Calloway et al. 2000) that may concur to the well-known age-related OXPHOS decline (Shigenaga et al. 1994). Mitochondrial involvement in AD and mtDNA heteroplasmy in brain tissues from AD patients has been extensively investigated (Bonilla et al. 1999; Lezza et al. 1999), but the role of mtDNA-inherited variability as a susceptibility factor in AD has been little explored (Chagnon et al. 1999). A reliable tool for investigating this point is the analysis of mtDNA haplogroups, continent-specific clusters of evolutionary-related mtDNA types that are defined on the basis of specific sets of stable ancestral associated polymorphic restriction sites (Ballinger et al. 1992). The analysis of mtDNA haplogroups is currently providing new in-

sights into the role of mtDNA-inherited variability in the quality of human aging (De Benedictis et al. 1999, 2000a, 2000b).

Starting from the pioneer work of Saunders et al. (1993), the role of apolipoprotein E (APOE) polymorphism (polymorphic sites at codons 112 and 158) as the susceptibility factor in sporadic AD has been confirmed in more than 90 papers published worldwide. In particular, the ε4 allele increases the risk (with a dose-effect in homozygosis) in all ethnic groups studied, across all ages between 40 and 90 years, and in both men and women (Farrer et al. 1997). However, variation at the APOE locus accounts only partially for the genetic variation in the liability to develop AD (Pericak-Vance and Haines 1995), and other polymorphisms could modulate the effect of APOE variability.

Oxidative damage and protection by antioxidants in the frontal cortex of AD patients are related to APOE genotypes (Ramassay et al. 1999). Furthermore, a relationship exists between APOE alleles and oxidative stress: the three apoE isoforms have allele-specific effects (E2>E3>E4) in protecting rat neuronal cell lines from oxidative cell death (Miyata and Smith 1996). Because of the allele-specific anti-oxidant effect of APOE and the role of the mitochondrial genome in OXPHOS, mtDNA haplogroups and APOE alleles may interact in the susceptibility for AD. In the present study, we explore this hypothesis by analyzing mtDNA haplogroups and APOE polymorphisms in a sample of sporadic AD patients genotyped for APOE and classified as APO ε4 carriers and non-carriers.

Materials and methods

Samples

A total of 213 sporadic AD patients (NINCDS-ADRDA diagnostic criteria, McKhann et al. 1984) with an onset age of more than 50 years (median age: 62 years; minimum: 51, maximum: 82; 85 males and 127 females) was analyzed. All patients had been investigated over three generations in order to exclude affected relatives (1st and 2nd degree). Complete clinical assessment, neuropsychological tests, neuroradiological examination, laboratory tests (B12; folate; syphilis serology; lipidemic parameters) had been carried out in order to exclude other possible causes of dementia. Two control groups were also analyzed. Group A comprised 179 healthy volunteer donors (median age: 64 years; minimum 50, maximum 79; 114 males and 65 females). Group B comprised 210 subjects older than 100 years, free from clinically manifest diseases and in relatively good health (61 males and 149 females). Specimens from Group B had been selected for health status examination from a collection of more than 600 DNA samples of Italian centenarians and were from categories A and B as previously described (Franceschi et al. 2000). Both patients and controls shared ethnicity and geographic origin (Italy). Informed consent was obtained from each subject included in the study.

DNA analyses

After DNA extraction from blood buffy coats, APOE ε2, ε3, and ε4 alleles were identified by a polymerase chain reaction (PCR) amplification refractory mutation system (Wenham et al. 1991). The primers given by Wenham et al. (1991) were used to identify the Arg/Cys polymorphism at codon 112 of the APOE gene, whereas new primers were used to identify the Arg/Cys polymorphism at codon 158 of the APOE gene. In particular, the APOE-a sense primer 5'atgccgatgacctgcagaggc3' and APOE-b sense primer 5'atgccgatgacctgcagaggt-3' with APOE-f1 antisense primer 5'-gtccggctgcccatctcctc-3' were used to identify Arg_{158}/Cys_{158}. MtDNA haplogroup typing was carried out by restriction analyses according to Torroni et al. (1996). Briefly, mtDNA specific primers were used to amplify by PCR the mtDNA regions containing the polymorphic restriction sites that characterize each European haplogroup, and the amplified fragments, after digestion by appropriate restriction enzymes (see Table 1), were separated by agarose gel electrophoresis. By this procedure, each mtDNA was ascribed to one of the nine haplogroups (H, I, J, K, T, U, V, W, X) specific to Europeans. Rare haplogroups (I, V, W, X) and mtDNA that were non-classifiable within a haplogroup were grouped as "Others" (Torroni et al. 1996). Detailed protocols regarding APOE and mtDNA genotyping are available on request.

Statistical analyses

APOE allele frequencies were estimated by counting genes from the observed genotypes. The Hardy-Weinberg equilibrium (HWE) was tested by shuffling (10,000 random allele permutations for each sample; Weir 1996). Standard errors for both alleles and haplogroups were computed according to the hypothesis of the multinomial distribution.

Permutation tests were also used to verify the null hypothesis of homogeneity between groups. Fisher exact tests were used to verify whether the frequency of a specific variable was different between samples. When the frequency of a single mtDNA haplogroup was considered, the level of significance was reduced to $\alpha=1\text{-}0.95^{1/5}=0.010$ (five independent variables) for multiple comparisons. The 95% confidence intervals (c.i.) of odds ratios (OR) were calculated by the bootstrap method.

Table 1 Identification of mtDNA haplogroups by restriction analysis (*a Alu*I, *b Ava*II, *c Dde*I, *g Hin*fI, *m Bam*HI, *n Hae*II, *q Nla*III, *p Bsto*I, *1/0* presence/absence of the restriction site, *#* associations of sites that characterize each haplogroup) of the specific target sequence (Torroni et al. 1996). Sites are numbered from the first nucleotide of the restriction site according to Anderson et al. (1981)

Haplogroup	Polymorphic restriction sites									
	1715c	4577q	7025a	8249b	9052n	10394c	12308g	13366m	13704p	16389m
H	1	1	0#	0	1	0#	0	0	1	0
I	0#	1	1#	1#	1	1#	0	0	1	1#
J	1	1	1#	0	1	1#	0	0	0#	0
K	1	1	1#	0	0#	1#	1#	0	1	0
T	1	1	1#	0	1	0#	0	1#	1	0
U	1	1	1#	0	1	0#	1#	0	1	0
V	1	0#	1#	0	1	0#	0	0	1	0
W	1	1	1#	1#	1	0#	0	0	1	0
X	0#	1	1#	0	1	0#	0	0	1	0

Table 2 APOE genotypes and alleles in AD patients, age-matched controls (Control Group A) and centenarians (Control Group B). Observed and expected (under HWE) absolute frequencies are shown for genotypes. Absolute and relative (in %±standard error) frequencies are shown for alleles

APOE Genotypes	AD patients		Control Group A		Control Group B	
	Observed	Expected	Observed	Expected	Observed	Expected
2.2	1	0.26	1	1.02	1	0.69
2.3	12	10.56	22	22.40	21	21.66
2.4	1	3.91	3	2.56	1	0.97
3.3	106	105.63	125	123.20	171	171.00
3.4	76	78.17	25	28.21	16	15.34
4.4	17	14.47	3	1.61	0	0.34
Total	213	213	179	179	210	210
Allele	Frequency (%±SE)		Frequency (%±SE)		Frequency (%±SE)	
2	15 (3.5±0.9)		27 (7.5±1.4)		24 (5.7±1.1)	
3	300 (70.4±2.2)		297 (83.0±2.0)		379 (90.2±1.4)	
4	111 (26.1±2.1)		34 (9.5±1.5)		17 (4.1±1.0)	
Total	426		358		420	

Table 3 Absolute frequencies of mtDNA haplogroups in AD patients, age-matched controls (Control Group A) and centenarians (Control Group B), stratified according to the presence/absence of the ε4 allele in the genotype. The relative frequency (×100 ± standard error) of each haplogroup in the samples is given in *parentheses*

MtDNA haplogroup	AD patients			Control Group A			Control Group B		
–	ε4+	ε4–	Total (%+SE)	ε4+	ε4–	Total (%+SE)	ε4+	ε4–	Total (%+SE)
H	42	50	92 (43.2±3.4)	14	47	61 (34.1±3.5)	5	78	83 (39.5±3.4)
J	11	7	18 (8.5±1.9)	1	13	14 (7.8±2.0)	3	20	23 (11.0±2.2)
K	7	5	12 (5.6±1.6)	4	13	17 (9.5±2.2)	–	15	15 (7.1±1.8)
T	18	15	33 (15.5±2.5)	6	15	21 (11.7±2.4)	3	15	18 (8.6±1.9)
U	4	20	24 (11.3±2.2)	3	17	20 (11.2±2.4)	2	24	26 (12.4±2.3)
[a]Others	12	22	34 (16.0±2.5)	3	43	46 (25.7±3.3)	4	41	45 (21.4±2.8)
Total	94	119	213 (100.0)	31	148	179 (100.0)	17	193	210 (100.0)

[a]Rare haplogroups (I, V, W, X) and mtDNA non-classified within a haplogroup are grouped as *Others* (Torroni et al. 1996)

Results

No significant differences for the APOE polymorphism or for mtDNA haplogroups were found between sexes within each sample (data not shown). Males and females were therefore combined to increase the statistical power in the following analyses. Data for the APOE polymorphism in AD patients, and Control Groups A and B are shown in Table 2. In all the samples, the observed genotypes fitted with those expected at HWE (P=0.127 in the AD sample; P=0.458 in Control Group A; P=0.746 in Control Group B). A highly significant difference was found between the frequency distributions in patients and Control Group A ($P<1\times10^{-4}$ both for genotypes and alleles), and in patients and Control Group B ($P<1\times10^{-4}$ both for genotypes and alleles). The frequency of the rare allele ε2 was almost the same in the three groups, whereas ε3 increased and ε4 decreased from AD patients to centenarians (Table 2). Furthermore, a significant difference was found between Groups A and B (P=0.031 for genotypes and P=0.005 for alleles), thus confirming, in Italians, the APOE-longevity association observed in French (Schachter et al. 1994) and Finnish (Louhija et al. 1994) populations. The OR of 0.42 (95% c.i. 0.21–0.78), estimated by using Group A as controls and Group B as cases, confirmed that the ε4 allele is unfavorable to longevity.

Subsequently, the distribution of mtDNA haplogroups in carriers and non-carriers of the ε4 allele was examined in each sample group (Table 3). In AD patients, the whole distribution of mtDNA haplogroups was significantly different between ε4 carriers and non-carriers (P=0.018). Furthermore, the frequency of the U haplogroup was lower in ε4 carriers than in non-carriers (4.3%±2.1% versus 16.8%±3.4%), and the observed frequency of individuals carrying both ε4 and U was 1.9% against a value of 5.0% expected on the hypothesis of random association (P=0.004 with α=0.01). The non-random association between APOE and mtDNA was restricted to AD patients. Indeed, no significant heterogeneity was found between ε4 carriers and non-carriers either in Control Group A (P=0.100) or in Control Group B (P=0.471), although the small number of ε4 carriers in these samples, and chiefly in Group B, lowered the power of the statistical test in both cases.

OR analyses were in agreement with the hypothesis of an interaction between the ε4 allele and mtDNA haplogroups in sporadic AD. By itself, the ε4 allele yielded an OR of 3.77 (95% c.i. 2.40–6.20) with respect to the age-matched Control Group A. When mtDNA haplogroups

Table 4 OR referring to ε4 carrier AD patients having different mtDNA haplogroups[a] estimated with respect to control group A. The 95% confidence interval (*c.i.*) is given in *parentheses*

	Control Group A
Haplogroups	OR (95% c.i.)
H	2.895 (1.615–6.104)
J	9.693 (1.850–15.819)
K	1.487 (0.406–7.020)
T	2.662 (1.099–9.689)
U	1.123 (0.204–4.841)

[a]The haplogroup category indicated as "Others" was not analyzed, since it represents a heterogeneous group of several mtDNA types (Torroni et al. 1996) and includes the rare haplogroups I, V, W, X

were considered together with the ε4 allele, OR for specific ε4/mtDNA combinations became non-significant, as reported in Table 4 (haplogroups K and U).

Discussion

Based on the allele-specific antioxidant capability of APOE and the biological role of the mitochondrial genome, the present study has examined whether mtDNA haplogroups and the ε4 allele are randomly associated in sporadic AD. A different distribution of mtDNA haplogroups has been found between ε4 carriers and non-carriers in AD patients; this is not present either in age-matched controls or in centenarians. This finding suggests that some haplogroups are able to modulate susceptibility to the disease. Indeed, some haplogroups seem to neutralize the harmful effect of the ε4 allele by lowering OR to non-significant values (K and U in Table 3). The statistical interaction observed between APOE alleles and mtDNA haplogroups in AD could be related to the antioxidant role exerted by APOE. It has recently been shown that mtDNA haplogroups are significantly associated with OXPHOS performance (Ruiz-Pesini et al. 2000), and that OXPHOS performance is correlated with reactive oxygen species (ROS) production (Esposito et al. 1999). We can speculate that the low antioxidant effect of the E4 isoform with respect to E2 and E3 is compensated by a moderate ROS production by mtDNA types included in haplogroups K and U. In this connection, it is worth noting that the K and U haplogroups share several variants, and that K is one of the U sub-clades (Macaulay et al. 1999). Measurements of OXPHOS and ROS in hybrid cell lines having various combination of mtDNA types and APOE genotypes may help to check the above hypothesis. In any case, as can be verified from Table 3, mtDNA haplogroup frequencies considered independently from APOE polymorphism do not differentiate AD patients from controls. This implies that mtDNA-inherited variability is not per se an AD susceptibility factor but could influence the effect of susceptibility factors such as ε4. This consideration confirms that AD is expressed following complex biological interactions between several genetic factors, which in turn may modulate the role of environmental determinants, such as head injury, stroke, or hypoxia (Roses 1997).

In conclusion, the data presented here show that APOE and mtDNA polymorphisms are statistically dependent variables in AD, whereas they are independent in groups of subjects free from the disease. Future molecular experiments in vitro, e.g., the construction of hybrid cells, may explain the putative biochemical mechanism of such statistical interactions.

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References

Anderson S, Bakier AT, Barrel BG, Bruijin MLH de, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Ballinger SW, Schurr TG, Torroni A, Gan YY, Hodge JA, Hassan K, Chen KM, Wallace DC (1992) South-east Asian mitochondrial DNA analysis reveals genetic continuity of ancient Mongoloid migrations. Genetics 130:139–152

Blass JP, Gibson GE (1991) The role of oxidative abnormalities in the pathophysiology of Alzheimer's disease. Rev Neurol 147: 513–525

Bonilla E, Kurenai T, Hirano M, Tuan HV, Di Mauro S, Schon EA (1999) Mitochondrial involvement in Alzheimer's disease. Biochim Biophys Acta 1410:171–182

Calloway CD, Reynolds RL, Herrin GL, Anderson WW (2000). The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. Am J Hum Genet 66:1384–1397

Chagnon P, Gee M, Filion M, Robitaille Y, Belouchi M, Gavreau D (1999) Phylogenetic analysis of the mitochondrial genome indicates significant differences between patients with Alzheimer disease and controls in a French-Canadian founder population. Am J Med Genet 85:20–30

De Benedictis G, Rose G, Carrieri G, De Luca M, Falcone E, Passarino G, Bonafè M, Monti D, Baggio G, Bertolini S, Mari D, Mattace R, Franceschi C (1999) Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans. FASEB J 13:1532–1536

De Benedictis G, Carrieri G, Varcasia O, Bonafè M, Franceschi C (2000a) Inherited variability of the mitochondrial genome and successful aging in humans. Ann N Y Acad Sci 908:208–218

De Benedictis G, Carrieri G, Garasto S, Rose G, Varcasia O, Bonafè M, Franceschi C, Jazwinski SM (2000b) Does a retrograde response in human aging and longevity exist? Exp Gerontol 35:795–801

Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC (1999) Mitochondrial disease in mouse results in increased oxidative stress. Proc Natl Acad Sci USA 96:4820–4825

Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH Pericak-Vance MA, Risch N, Duijn CM van (1997) Effects of age, sex and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA 278:1349–1356

Franceschi C, Motta L, Italian Multicenter Study on Centenarians (IMUSCE) (2000) Do men and women follow different trajectories to reach extreme longevity? Aging Clin Exp Res 12:77–84

Gibson GE, Sheu KF, Blass JP (1998) Abnormalities of mitochondrial enzymes in Alzheimer's disease. J Neural Transm 105: 855–870

Lezza AMS, Mecocci P, Cormio A, Flint-Beal M, Cherubini A, Cantatore P, Senin U, Gadaleta MN (1999) Mitochondrial DNA 4977 bp deletion and OH^8dG levels correlate in the brain of aged subjects but not Alzheimer's disease patients. FASEB J 13:1083–1088

Louhija J, Miettinen HE, Kontula K, Tikkanen MJ, Miettinen TA, Tilvis RS (1994) Ageing and genetic variation of plasma apolipoproteins. Relative loss of the apolipoprotein E4 phenotype in centenarians. Arterioscler Thromb Vasc Biol 14:1084–1089

Macaulay V, Richards M, Hickey E, Vega E, Cruciani F, Guida V, Scozzari R, Bonnè-Tamir B, Sykes B, Torroni A (1999) The emerging tree of West Eurasian mtDNAs: a synthesis of control-region sequences and RFLPs. Am J Hum Genet 64:232–249

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Service Task Force on Alzheimer's disease. Neurology 34:939–944

Miyata M, Smith JD (1996) Apolipoprotein E allele-specific antioxidant activity and effects on cytoxicity by oxidative insults and β-amyloid peptides. Nat Genet 14:55–61

Pericak-Vance MA, Haines JL (1995) Genetic susceptibility to Alzheimer disease. Trends Genet 11:504–508

Ramassamy C, Averill D, Beffert U, Bastianetto S, Theroux L, Lusier-Cacan S, Cohn JS, Christen Y, Davignon J, Quirion R, Poirier J (1999) Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. Free Radic Biol Med 27:544–553

Roses AD (1997) Apolipoprotein E, a gene with complex biological interactions in the aging brain. Neurobiol Dis 4:170–185

Ruiz-Pesini E, Lapeña AC, Díez-Sánchez C, Pérez-Martos A, Mantoya J, Alvarez E, Díaz M, Urriés A, Montoro L, López-Pérez MJ, Enríquez JA (2000) Human mtDNA haplogroups associated with high or reduced spermatozoa motility. Am J Hum Genet 67:682–696

Saunders AM, Strittmatter WJ, Schmechel D, St.George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ, et al (1993) Association of apolipoprotein E allele ε4 with late-onset familial and sporadic Alzheimer's disease. Neurology 43:1467–1472

Schachter F, Faure-Delaneff L, Guenot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D (1994) Genetic associations with human longevity at the APOE and ACE loci. Nat Genet 6: 29–32

Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA 91:10771–10778

Shoffner JM (1997) Oxidative posphorylation defects and Alzheimer's disease. Neurogenetics 1:13–19

Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, Obinu D, Savontaus ML, Wallace DC (1996) Classification of European mtDNAs from an analysis of three European populations. Genetics 144:1835–1850

Wallace DC (1994) Mitochondrial DNA sequence variation in human evolution and disease. Proc Natl Acad Sci USA 91:8739–8746

Wenham PR, Newton CR, Price WH (1991) Analysis of apolipoprotein E genotypes by the amplification refractory mutation system. Clin Chem 37:241–244

Weir BS (1996) Genetic data analysis II. Sinauer Associates, Sunderland, Mass., USA, pp 108–110