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Ph.D. in Molecular Bio-pathology

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Analysis of C538T polymorphism of the SSADH gene in humans: functional and evolutionary aspects

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SOMMARIO

I polimorfismi dei geni umani che codificano per molecole neurologiche hanno suscitato un notevole interesse scientifico a causa delle conseguenze fenotipiche che essi possono avere. Infatti, la variabilità di questi geni può dare origine sia a fenotipi patologici che a piccole ma importanti variazioni delle capacità cognitive e comportamentali. Inoltre diversi studi hanno evidenziato che i geni codificanti per molecole neurologiche possono essere estremamente interessanti dal punto di vista evolutivo. Infatti, poiché essi sono coinvolti nella determinazione dell'eccezionale complessità del cervello dell'*Homo sapiens sapiens*, la variabilità della loro sequenza può aiutare sia a spiegare le differenze comportamentali con gli altri primati sia a comprendere l'evoluzione di diversi tratti umani.

La succinico semialdeide deidrogenasi (SSADH) è un enzima mitocondriale NAD+-dipendente la cui deficienza causa una patologia neurologica eterogenea, la 4-idrossibutirrico-aciduria (4-HBA). L'analisi della variabilità del gene *SSADH* ha portato all'identificazione di numerose varianti nucleotidiche, sia negli esoni che negli introni del gene. La variante più polimorfica è risultata essere la sostituzione C>T alla posizione 538 del cDNA del gene *SSADH*, che comporta la sostituzione aminoacidica istidina-tirosina alla posizione 180 del peptide corrispondente con importanti effetti sulla funzionalità dell'enzima. Infatti, la proteina codificata dall'allele 538T (Tyr_{180}), risulta essere l'82% dell'attività della proteina codificata dall'allele 538C (His_{180}). Studi evolutivi hanno permesso di identificare l'allele 538T come ancestrale e hanno evidenziato che l'allele C è presente solo nell'uomo, poiché tutti i primati condividono l'allele T. La differenza dell'attività enzimatica tra i due alleli ha importanti effetti a livello fenotipico. In particolare, un recente studio ha mostrato che l'allele C è associato con un aumento delle capacità cognitive nella popolazione generale.

Durante il mio Dottorato di Ricerca mi sono occupata dello studio di due diversi aspetti correlati agli effetti fenotipici del polimorfimo C538T: i) gli effetti del polimorfismo sulla conservazione della funzione cognitiva nell'anziano; ii) la distribuzione globale nelle popolazioni umane dei due alleli del polimorfismo per ricercare eventuali segnali di selezione.

Lo studio dell'associazione tra il polimorfismo C538T e la conservazione della funzione cognitiva nell'anziano è stato effettuato in un campione di popolazione di età compresa tra 65 e 85 anni. Abbiamo osservato che il genotipo TT è sovra-rappresentato in un sottocampione di soggetti con funzione cognitiva deteriorata rispetto ad un gruppo di soggetti con buona conservazione della funzione cognitiva. Poiché la funzione cognitiva è fondamentale per l'aspettativa di vita nell'anziano, abbiamo successivamente studiato gli effetti del polimorfismo sulla mortalità in un campione più ampio della stessa popolazione. Il genotipo TT è risultato influenzare la sopravvivenza al di sopra dei 65 anni di età. Infatti, dopo questa età, la funzione di sopravvivenza dei soggetti omozigoti TT è più bassa di quella degli individui con genotipo CC e CT.

La nostra analisi sulla distribuzione delle frequenze del polimorfismo C538T nelle popolazioni umane ha mostrato un chiaro pattern geografico delle varianti *SSADH* analizzate, con frequenze più alte dell'allele ancestrale 538T in Africa, in contrasto con alte frequenze dell'allele derivato 538C fuori dell'Africa. Abbiamo inoltre analizzato, nelle popolazioni umane, la co-variazione del polimorfismo C538T *SSADH* con un altro marker per il quale è stata riportata una forte evidenza di selezione naturale positiva, la microcefalina (MCPH1), ed abbiamo osservato una correlazione significativa tra le frequenze degli alleli derivati nei due geni. Questi risultati sono in accordo con l'ipotesi di una selezione naturale positiva sull'allele 538C del gene *SSADH*.

Nell'insieme, i nostri risultati confermano che il polimorfismo comune C538T del gene *SSADH* ha importanti effetti non neutrali sul fenotipo cognitivo nell'uomo e ciò sembra aver implicato una selezione naturale positiva su uno dei due alleli all'interno delle popolazioni umane.

SUMMARY

Polymorphisms of the human genes whose products are involved in the metabolism of neuroactive molecules are interesting because of the phenotypic consequences of their variability. In fact, the variability of these genes, which are often involved in behavioural and cognitive functions, may lead to pathological phenotypes or to subtle but important variations of the cognitive and behavioural performances. In addition, it is emerging that these genes encoding for neurological molecules may be extremely interesting from an evolutionary point of view. In fact, since they are involved in determining the exceptional complexity of the *Homo sapiens sapiens* brain, their sequence variability may help both to explain the behavioural differences with other primates and to understand the evolution of different human traits .

Mitochondrial NAD+-dependent succinic semialdehyde dehydrogenase (*SSADH*) is a gene which is supposed to contribute to cognitive performance in humans. It is a gene of medical interest, because the enzyme deficiency causes an heterogeneous neurological disease, 4-hydroxybutyric aciduria (4-HBA). The analysis of the common variability of *SSADH* gene lead to the identification of many nucleotidic variants, both exonic and intronic. The most polymorphic variant was found to be the C>T substitution at the position 538 of *SSADH* cDNA, leading to a histidine-tyrosine substitution at aminoacid position 180 of the native polypeptide. Interestingly, the enzymatic activity of the protein encoded by the allele 538T (Tyr_{180}), resulted to be 82.5 % of the activity of the protein encoded by the allele 538C (His_{180}). Evolutionary studies allowed to identify the allele 538T as the ancestral state and that the C allele is specific of humans, as all the other primates share the ancestral T allele. The difference of the enzymatic activity between the two alleles has important effects at the phenotypic level. In particular, a recent study showed that the C allele was associated with increased cognitive ability in the general population.

During my PhD appointment I was involved in the study of two different aspects correlated to the phenotypic effects of *SSADH* C538T polymorphism: i) the effects of the polymorphism on the preservation of the cognitive ability in the elderly; ii) the global

distribution in the human population of the two alleles of the polymorphism to find out any signature of non neutral evolution.

The association between the C538T polymorphism and preservation of cognitive function in the elderly was studied by surveying a population sample aged 65-85 years from southern Italy. We found that the TT genotype is overrepresented in subjects with impaired cognitive function compared to those with conserved cognitive function. Then, as cognitive function is crucial for survival chance, we studied the effects of this polymorphism on survival in a larger sample aged 18-107 years from the same population. We found that the TT genotype affects survival after 65 years of age. In fact, after this age, the survival function of TT homozygous subjects is lower with respect to CT and CC subjects.

The assay of worldwide frequencies of C538T polymorphism in human populations showed a clear geographic pattern of analyzed *SSADH* variants, with high frequencies of the ancestral allele C in Africa, contrasting with high frequencies of the derived variant (allele T) out of Africa. We explored the covariation across human populations with another marker for which strong evidence of positive selection was produced, i.e. MCPH1. We found a significant correlation between the frequencies of the derived alleles in the two genes. These data are in agreement with the hypothesis of a positive natural selection on allele 538C of *SSADH* gene.

On the whole, our results confirm that the common polymorphism C538T of *SSADH* gene has important non neutral effects on the human cognitive phenotype and this seems to have implied a positive selection on one of the two alleles within the human populations.

List of abbreviations

AD	Alzheimer's disease
ALDH	aldehyde dehydrogenase
cDNA	complementary DNA
CNS	central nervous system
DHHA	4,5-dihydroxy-hexanoic acid
Dn/Ds	nonsynonimous versus synonimous
ECHA	European challenge for healthy ageing
EEG	electroencephalogram
EST	expressed sequence tag
FOXP2	forkhead box P2
GABA	γ-aminobutirric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GD	genetic-demographic
GHB	γ-hydroxybutyrate
4-HBA	4-hydroxybutyric aciduria
4-HBAD	4-hydroxybutyrate-dehydrogenase
HNE	4-hydroxy-2-nonenal
IQ	intelligence quotient
MCPH1	microcephalin
MMSE	mini mental state examination
NAD(P)	nicotinamide adenine dinucleotide phosphate
ORF	open reading frame
PCR	polymerase chain reaction
RD	reading disability
RT-PCR	reverse transcriptase polymerase chain reaction
SNP	single nucleotide polymorphism
SSA	succinic semialdehyde

SSADH	succinic semialdehyde dehydrogenase
TDT	transmission disequilibrium test
vigabatrin	γ-vinil GABA
YAC	yeast artificial chromosome

1. INTRODUCTION

In the recent years there has been a growing interest for the variability of genes which are involved in the metabolism of neurological molecules. Such interest is primarily due to the phenotypic consequences of the variability of these genes, which may lead, to pathological or impaired phenotypes. On the other hand, the variability of neurological molecules, due to the sensitivity of their functions and of their phenotypic effects, turned out to be extremely interesting also from an evolutionary point of view. In fact, they shed light on some important aspects of human evolution. In this frame, succinic semialdehyde dehydrogenase (*SSADH; ALDH5A1*) turned out to be one of the interesting genes. In fact variability of *SSADH* turned out to affect neurological phenotypes. In addition, data on interspecific divergence among primates, showed that this gene is highly conserved and confirmed it is essential for brain metabolism.

SSADH is a mitochondrial NAD^+-dependent enzyme (EC 1.2.1.24) belonging to the aldehyde dehydrogenase (ALDH) superfamily, a related group of enzymes that metabolize a wide spectrum of aldehydes in the corresponding carboxylic acids, by a NAD(P) dependent reaction essentially irreversible. Aldehyde dehydrogenases are thought to be detoxifying enzymes that eliminate exogenous and endogenous aldehydes, the latters coming from the metabolism of aminoacids, alcohol, vitamins, steroids and lipids. The presence of ALDH encoding genes in the majority of bacterial and eukaryotic genomes suggests that these enzymes play a very important role in metabolic processes and that the ALDH superfamily has an ancient origin. Up to now 17 ALDH functional genes and 3 pseudogenes have been identified in the human genome (Vasiliou et al., 1999; Sophos et al., 2003). Some peptide regions are conserved in all or most of the ALDHs, and they are thought to be functionally constrained (Yoshida et al., 1998).

SSADH is the final enzyme in the catabolism of γ-aminobutyric acid (GABA) (**Fig.1**), the major inhibitory neurotransmitter of the mammalian central nervous system (Waagepetersen. et al., 1999).

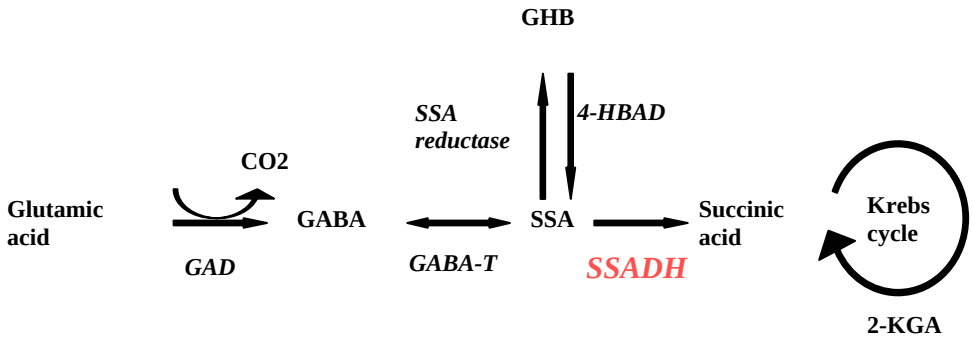

Fig 1. *Metabolic GABA pathway (Blasi et al., 2002)*

SSADH is localized into neurons of the central nervous system, where it is primarily responsible for the oxidation of succinic semialdheyde (SSA), originated by GABA deamination, to succinic acid.

In addition to the role played in the GABA metabolism, SSADH may also play a role in the oxidation and removal of other toxic aldehydes in the brain (Picklo et al., 2001a; Picklo et al., 2001b). In agreement with this hypothesis it has been demonstrated that in the rat central nervous system SSADH catalyses the oxidation of reactive substances other than SSA, such as 4-hydroxy-2-nonenal (HNE) a neurotoxic aldehyde product of lipid peroxidation that is implicated as toxic "second messenger" of oxidative damage in numerous neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease (Murphy et al. 2003; Meyer et al., 2004).

The functional importance of SSADH becomes well evident when the enzyme doesn't work properly; in fact SSADH deficiency causes a metabolic disorder in the central nervous system that gives rise to a pathological neurological phenotype, 4-hydroxybutyric aciduria (4-HBA) (OMIM 271980). The majority of patients display delayed intellectual, motor, speech and language development as the most common manifestations, associated with hypotonia, hyporreflexia, seizures and a non progressive ataxia. Pervasive developmental delay and obsessive-compulsive disorders

have been observed, which are core clinical features of the autism spectrum. Such disorders result from the impairment of the metabolic pathways where SSADH has a central role, in particular from the imbalance of GABA and GHB neurotrasmitters which play a central role in modulating many crucial functions of the brain. The pathology is heterogeneous both because many mutations have been demonstrated to be causative of the disease and because different degrees of seriousness have been observed. This has suggested that normal variability of the catalytic activity levels of SSADH enzyme may be correlated with the common non-pathological variability of cognitive phenotypes. These considerations stimulated the beginning of a systematic exploration of normal nucleotide variability in human populations, and this led to the identification of several polymorphisms (Akaboshi et al., 2001). Consequently, many studies have been carried out to figure out the correlation between the SSADH variability and human cognitive phenotypes and to find out if, due to their phenotypic effects, any nucleotide variant has been favoured along human evolution (Blasi et al., 2006).

The gamma aminobutyric (GABA) metabolism

Gamma aminobutyric acid (GABA) is a nonprotein four-carbon aminoacid found in all prokaryotic and eukaryotic organisms. It also exists in the form of a dipeptide with histidine, known as homocarnosine. GABA is produced via decarboxylation of L-glutamate by glutamic acid decarboxylase (GAD). There are two isoforms of GAD (GAD_{65} and GAD_{67}) with different subcellular distribution and regulatory properties. The major precursor of glutamate, glutamine, is synthesized in glial cells and transported to neurons, where glutamate and GABA are metabolized. In the brain GABA is synthesized in presynaptic nervous endings, packaged into vescicles via Mg^{2+}-activated ATPase, released into the synaptic cleft and bound by specific postsynaptic receptors that elicit the inhibition of neurotransmission (**Fig. 2**).

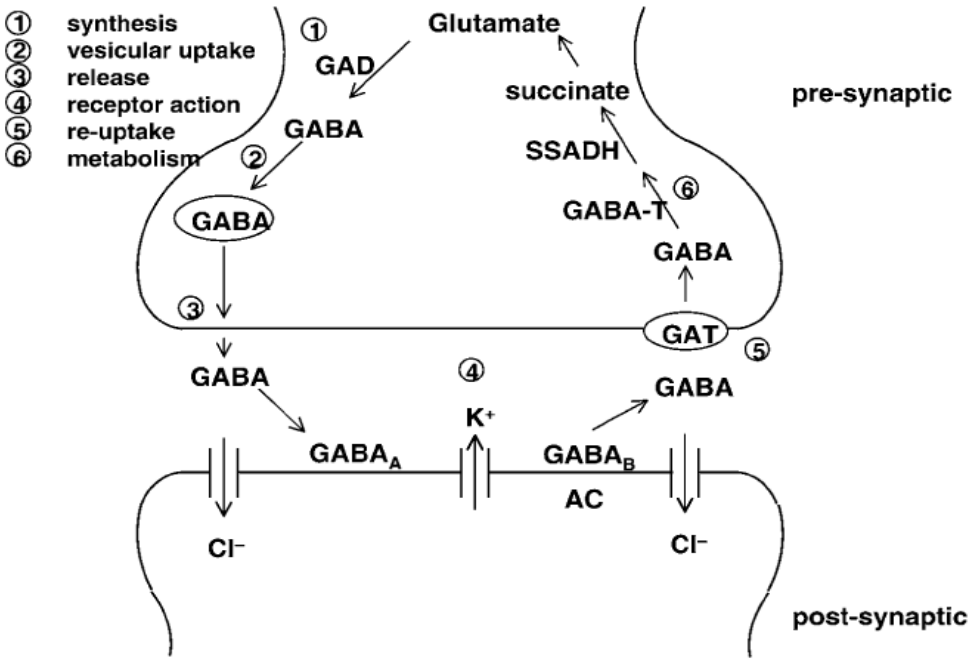

Fig. 2. *The GABAergic synapse (Gibson, 2005).*

In synapses there is no enzyme able to inactivate or degradate GABA. After its release from the receptors, GABA is reabsorbed by glial cells and presynaptic neurons, where degradation takes place; synaptic removal occurs via the GABA transporter (GAT). GABA is then recycled via breakdown to metabolites eventually used in its resynthesis, which constitutes the GABA shunt (Tillakaratne et al., 1995; Gibson, 2005).

GABA is not confined to the brain, but it is widely distributed also in nonneural tissues including peripheral nervous system, endocrine and several other tissues where it may play different physiologic roles (Tillakaratne et al., 1995). In neural and nonneural tissues, three enzymes are involved in its degradation (Fig.1). The first enzyme, GABA-transaminase (GABA-T), metabolizes GABA to the corresponding aldehyde, the succinic semialdehyde (SSA), by a reversible reaction.

Two reactions can alternatively transform the SSA in succinate or γ-hydroxy butyrate (GHB). The first reaction is an irreversible oxidization that takes place in mithocondrions by NAD-dependent SSADH. In the brain the production of succinic acid by SSADH allows entry of the GABA carbon skeleton into the tricarboxylic acid cycle. The ongoing conversion of glutamate to GABA and then back to glutamate is

known as “GABA shunt”. This pathway is important in brain mitochondrions, linking the metabolism of the major transmitters glutamate and GABA to energy production; the overall contribution to the Kreb’s cycle through this way is about 15%. Succinic semialdehyde, the substrate of SSADH, can also undergo export from the mithocondrion, probably by a regulated transport mechanism. In the cytosol, the at least two NADPH-dependent enzymes are responsible for its reduction to γ-hydroxy butyrate (GHB), (Cash et al., 1979). The 4-hydroxybutyrate-dehydrogenase (4-HBAD) can transform again γ-hydroxy butyrate in succinic semialdeide .

GABA action

Gamma aminobutyric acid is primarily an inhibitory neurotransmitter in the CNS. Up to one third of synapses utilize GABA. This function is mediated by $GABA_A$, $GABA_B$, and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are both chloride channels regulators. $GABA_B$ receptors are essentially presynaptic, usually coupled to potassium or calcium channel, and they act via a GTP binding protein (Gibson, 2005). GABA transporters have been identified on neuron and astrocytes, but this aminoacid is taken out predominantly by neurons. GABA is distributed in the brain among distinctly different cellular pools, possibly reflecting multiple functions other than neurotransmitter, probably as metabolite and neurotrophin (Wolff et al., 1978; Waagepetersen et al., 1999).

GABA receptors have been detected in many nonneural tissues like smooh muscle cells (blood vessels, gut, oviduct, uterus, seminal vesicles, prostate gland and urinary bladder), epithelial cells (exocrine pancreas, stomach, seminal vescicle, prostate gland), hepatocytes and germ cells. The presence of GABA, its receptors and its metabolic enzymes in nonneural tissues supports the hypothesis that GABA may play a multitude of functions in these tissues. For example, GABA may be important in local regulation of seminal function (Kerr and Ong, 1992), and in the control of hormone release in endocrine cells of pancreas, adrenal medulla and gastrointestinal tract (Gilon and Remacle, 1992).

GHB action

Gamma-hydroxy butyric acid (GHB) is a short fat acid chain found in human brain; it is thought to be a neurotransmitter or neuromodulator (Cash, 1994), but its role is not completely clarified. GABA is the primary precursor of GHB in the brain.

GHB action is mediated by specific binding sites, but it can also interact with multiple receptor systems, including the $GABA_B$ receptors, the $GABA_A$-benzodiazepine receptor complex, and the opioid receptors.

When administered exogenously GHB is able to cross the blood-brain barrier and it can modulate the activity of various neurotransmitter systems, producing significant behavioural, electrophysiological, and biochemical effects (Snead, 2000). It has a variety of neuropharmacological and neurophysiologic effects which vary widely with dosage, including anxiolysis, sedation, anesthesia, changes in dopamine synthesis and release, and electroencephalographic alterations (Maitre., 1997). In the 1960s GHB was used as anaesthetic, but later its use was discarded because of its collateral effects. Because of its capacity to induce a state of perceived euphoria at low doses, perhaps related to alterations in GABA and dopamine release, GHB has been popularized as a drug of abuse; in the 1990s it was categorized as a drug, and its consumption has been described as associated with euphoria, respiratory depression, vomiting and even death. Recently the clinical use of GHB has been revaluated, and it is used in several applications like treatment of cataplexy, withdrawal syndromes related to alcohol and opiate addiction, treatment of fatal insomnia, and as cardiac tissue protectant (Thomas et al., 1997).

Effects of metabolic GABA pathway imbalance

The predominant pathway of succinic semialdehyde metabolism is the transformation in succinic aldehyde, because the Km of SSADH for SSA is 1-2μM, whereas the Km for the NADPH-dependent enzymes reducing SSA to GHB, ranges from 5 to 56μM. Thus, a balance exists between the amount of catabolites following one or the other of the two paths.

The disruptive effects of the imbalance in the endogenous production of GHB can be observed in patients affected by 4-hydroxybutyric aciduria (4-HBA), an autosomal recessive disorder due to SSADH deficiency (Jacobs et al., 1981). The disorder is typically a slowly progressive or static encephalopathy with late infantile to early childhood onset; although the disease is usually diagnosed in children it has been found in adults as well (Jacobs et al., 1990). In the enzyme deficient patients an accumulation of GABA and GHB in the central nervous system is found, causing a variety of cognitive, motor and developmental symptoms, ranging from mild retardation in psychomotor and language development to more severe neurological defects associated with hypotonia, abnormal reflexes, ataxia and behavioural problems, especially in older patients (Gibson et al., 1997). Nearly half of the patients have seizures, including absences as well as tonic-clonic convulsions (Gibson et al., 1983; Gibson et al., 2001; Pearl, 2003a). Electroencephalographic studies have disclosed a variety of background abnormalities and epileptiform features (Pearl et al., 2003b). Biochemically, patients show significant elevations of GHB in physiological fluids, facilitating detection using urine organic acid analysis. A proper diagnosis can be achieved by organic acid analysis of urine or plasma; enzyme analysis in leukocytes confirms the suspected diagnosis if the residual SSADH activity is very low (Gibson et al., 1994).

The pathogenic mechanisms leading to these features are not completely elucidated, but it is clear that the defect in SSADH enzyme blocks the oxidation of succinic semialdehyde, resulting in increasing concentration of GABA and in conversion of SSA to GHB, which accumulates in biological fluids and it seems to be the biochemical hallmark of this disorder. The medium concentration of GHB in patients cerebrospinal fluid is 526 $\pm$ 43 μM, more than 200 times the maximum value observed in normal subjects. The enzymatic block causes also an accumulation of GABA, with medium values about twice the superior threshold measured in normal controls. It is thought that GHB is the major neurotoxic agent causing brain damage. Its accumulation in CNS (central nervous system) may lead to an impairing of GABA-glutamate axis, resulting in an uncoupling of the normal balance between glutamatergic excitatory activity and GABAergic inhibition (Pearl et al., 2003b).

There is not effective treatment for SSADH deficiency. Therapeutic strategies include the use of vigabatrin (γ-vinil GABA), an antiepileptic agent that inhibits GABA transaminase and thus the formation of succinic semialdehyde and therefore GHB. Although vigabatrin leads to decreased GHB levels, there are increased GABA levels,

which would not be necessarily desirable. This has been the most widely utilized agent in the treatment of the disease, but long-term clinical efficacy is marginal. Furthermore vigabatrin is contraindicated due to retinal toxicity.

The murine model

Although the pathophysiology of SSADH deficiency remains unclear, the recent development of a mouse model is beginning to provide insights. The murine knock out model was obtained in 2001 (Hogema et al., 2001), and represent a valuable tool in understanding the relative roles of the different metabolites on 4-HBA, and also in elucidating the neurophysiological consequences of SSADH deficiency on other metabolic pathways.

The knock out mouse replicates most of the symptoms observed in human patients. Homozygous animals (*Aldh5a1*$^{-/-}$) fail to gain weight and to thrive; they show absence seizures, occurring as early as day of life 14, generalized convulsions at about 20-24 days of life, and they die at approximately day 30 (Cortez et al., 2004; Sauer et al., 2006). Like the human patients they show increased GHB and GABA level in body fluids and tissues.

The murine model also allowed to determine eventually additional biochemical perturbations beyond GHB and GABA. It was shown that in the CNS, significant GABA elevations are accompanied by reduced glutamine levels; glutamate levels were not abnormal (Gibson et al., 2002). This suggests disturbance of the glutamine/glutamate cycle which is important for the maintenance of transmitter pools between astrocytes and neurons, as well as for maintenance of energy homeostasis. Analysis of cerebrospinal fluid from human patients revealed low to borderline-low glutamine levels (Gibson et al., 2003).

The knock out mice shows a variety of biochemical abnormalities: increased alanine and decreased arginine concentrations in the CNS; increased SSA, homocarnosine (the GABA-histidine dipeptide) and guanidinobutyrate (which derives from GABA conjunction with the guanidine mojety of arginine) in the brain tissue (Gupta et al., 2004). For the majority of human patients with increased levels of GABA in cerebrospinal fluid, homocarnosine was also increased (Gibson et al., 2003).

In addition, increased levels of 4,5-dihydroxy-hexanoic acid (DHHA) a compound suggested to be derived from the condensation of SSA with a 2- or 3- carbon

intermediate (acetyl-CoA, lactate, pyruvate) (Schoerken and Sprenger 1998), have been found in brain homogenates of *Aldh5a1*$^{-/-}$ mice (Gibson et al., 2002) as well as in SSADH-deficient patients (Brown et al., 1987). DHHA has been shown to inhibit the respiratory chain function and antagonize the high-affinity GHB receptors, suggesting that it may play a role in the pathology (Okun et al., 2003).

The availability of a murine model has been very helpful in understaining of pathophysiology of SSADH deficiency (**Fig. 3**).

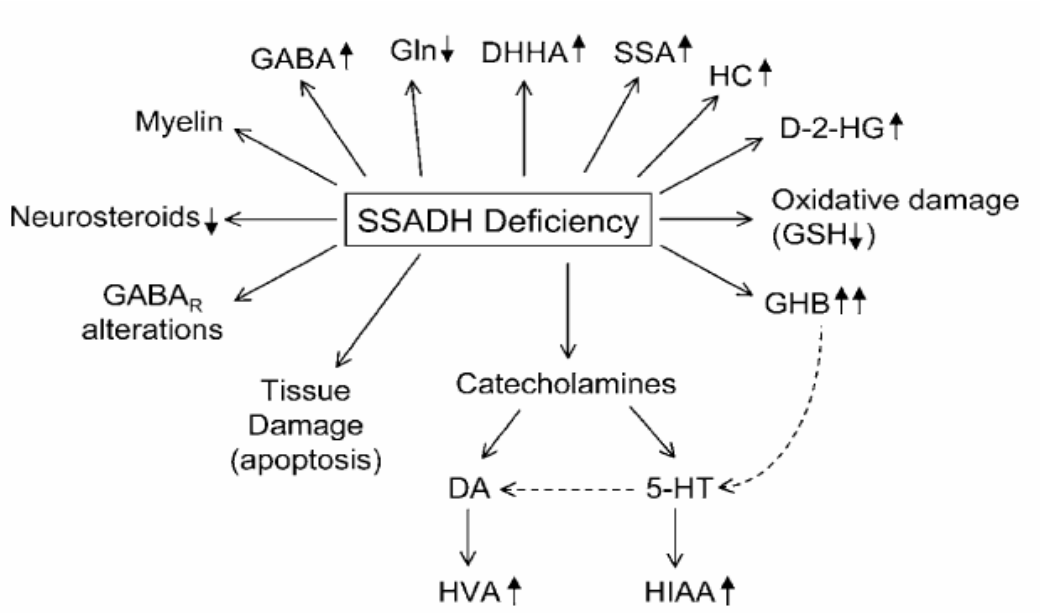

Fig. 3. *The state of knowledge about pathophysiological mechanism leading to 4-HBA features. Arrows indicate upward or downward pertubations as identified in either human or murine disease (Gibson, 2005).*

It is now clear that this enzyme deficiency is associated with many biochemical abnormalities; furthermore it has been shown that SSADH is involved in other biochemical pathways in addition to the role played in GABA metabolism. These

findings suggest that multiple physiologic ramifications leading to different mechanisms of neurotoxicity, can be involved in SSADH deficiency.

GHB, one of the two metabolites that accumulates in 4-hydroxybutyric aciduria, has traditionally been regarded as the neurotoxic agent leading to the clinical syndrome. It has been shown that when administered to animals and humans at pharmacologic levels GHB produces central nervous system effects, including altered motor activity and behaviour disturbances (Snead, 1978). In a recent work aimed at understanding the mechanism underlying the actions of GHB in the central nervous system, it was shown that GHB induce oxidative stress by stimulating lipid peroxidation and decreasing the non-enzymatic antioxidant defenses in cerebral cortex of young rats (Sgaravatti et al., 2007).

If this effect also occurs in humans, it is possible that oxidative damage may be one of the mechanisms contributing to the neurological dysfunction characteristic of SSADH deficiency.

The SSADH gene: structure and variability

SSADH is a highly conserved gene during evolution from bacterial to primate, sharing substantial homology even to E.coli gabD gene, a NADP+-dependent succinic semialdehyde dehydrogenase.

SSADH cDNAs were isolated based on the aminoacid sequence of SSADH protein, purified to apparent homogeneity from both rat and human brain (Chambliss and Gibson, 1992). Comparison between cDNAs and the inferred proteins from these two species showed an homology of 83% and 91% respectively (Chambliss et al., 1995). Northern-blot analysis demonstrated that the gene is expressed both in neural and non-neural tissue (Chambliss et al., 1995).

A complete structural analysis of SSADH protein is not yet available, partly because it is very difficult to obtain its crystal structure. Some experimental evidences indicate that the native purified protein has a homotetrameric structure, with each subunit having a molecular weight of 54,000-58,000 (Chambliss and Gibson, 1992). However, other Authors provided evidences of a SSADH protein with a

heterotetrameric structure, made of non-identical subunits of molecular weight 61,000-63,000 (Ryzlak and Pietruszko, 1988).

In humans the gene encoding SSADH has been mapped as a single-copy gene on chromosome 6p22 (Malaspina et al., 1996), a region representing 5-6% of the length of the whole chromosome in which many gene have been mapped such as prolactin gene, the histone 1 cluster, and several ESTs.

Human *SSADH* gene consists of 10 exons and 9 introns encompassing over 38 Kb (**Fig. 4**). The complete ORF was found to be of 1605bp encoding for 535 aminoacids, with the first 47 residues recognized as mitochondrial targeting peptide (Chambliss et al., 1998).

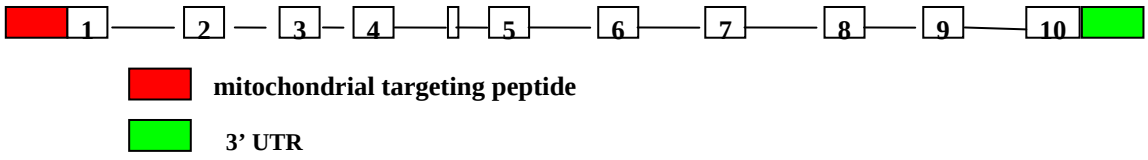

Fig .4. *Structure of the SSADH gene.*

Two differential expressed transcripts of approximately 6 and 2 Kb, referred to as *SSADH* L and S, were found both in human and rat (Chambliss et al., 1995). These two mRNA isoforms (5225 and 1827nt respectively) are due to alternative polyadenylation sites. Northern blot analysis of tissues revealed that the two transcripts are differentially expressed. Some tissues, like brain and pancreas preferentially express the L isoform and they show the S isoform in little amounts; other tissues, like heart, liver, skeletal muscle and kidney, have the two isoforms in similar amounts; no tissue expresses only the 2 Kb transcript.

As alternative polyadenylation is known to be a strategy for regulation of gene expression, the stability and the polysomal association of the two isoforms was tested; the two *SSADH* transcripts were found to be equally stable and to have the same translation efficiency in vitro (Blasi et al., 2002). However, the Authors do not exclude that the two isoforms may have preferential tissue or subcellular localization, or that

particular physiological conditions could cause the presence of one of the two isoforms.

Alternative splicing of SSADH mRNA

After the first report on the *SSADH* gene structure, an additional 39bp exon, (exon 4B), flanked by exons 4 and 5, was decribed (**Fig. 5**). This exon preserves an open reading frame and would code for additional 13 amino acids in the polypeptide. RT-PCR experiments showed that alternative splicing of exon 4B produces two mRNA isoforms other than the two major L and S isoforms, named isoform 1 and 2. These two isoforms are expressed in all cell lines and tissues examined, but their relative quantification is not known. It is not clear which of the possible combinations of isoforms 1 and 2 with the two major isoforms L and S (L plus or minus exon 4B, S plus or minus exon 4B) exist, nor if there are differences as it regards the ratio between different isoforms in different tissues (Blasi et al., 2002).

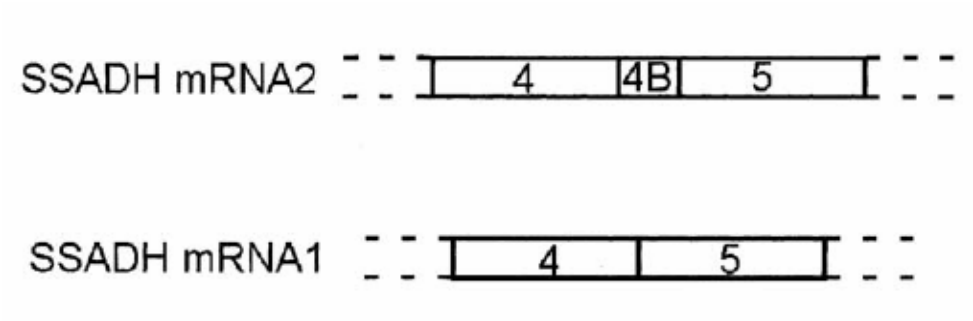

Fig. 5. *Alternative splicing of exon 4B (modified from Blasi et al., 2002).*

Promoter region and transcriptional start point

A computer analysis for promoter prediction in the 5'-UTR showed a high G + C content, the absence of TATA box and CAAT box and the presence of a putative promoter element 300bp immediately upstream of the ATG site. To experimentally characteryze the promoter, a series of recombinant plasmids containing different

portions of the *SSADH* 5'UTR was prepared and inserted upstream of the luciferase coding region. The plasmids were used in transient co-transfection experiments. The results showed the presence of multiple promoter elements within the 800bp region upstream of the ATG site, that regulate the transcription in a negative way (between -811 and -387) and in a positive way (between -387 and -184). A residual promoter activity is present between -184 and the single transcriptional start point of the gene, which was mapped at -122 by RNase protection assay (Blasi et al., 2002).

Pathological variability of SSADH gene

SSADH is a gene of medical interest, because of 4-hydroxybutyric aciduria (see above). From a genetic point of view, 4-hydroxybutyric aciduria is inherited as an autosomal recessive trait. Many mutations causing the deficiency have been identified (**Fig.6**). The spectrum of disease-causing mutations include point mutations (nonsense and missense, small insertions and deletions, and mutations affecting splice junctions. Some ins/del mutations cause a shift in the reading frame and give rise to a truncated polypeptide; single nucleotide substitutions are both transitions or transversions (De Vivo et al.,1988; Gibson et al.,1997; Chambliss et al., 1998; Akaboshi et al., 2001, ; Akaboshi et al., 2003, Bekri et al., 2004).

When a new mutation is found, the possibility that it represents a non-pathogenic polymorphism cannot be beforehand excluded. To confirm that the mutations found in 4-HBA patients are causative of enzymatic deficiency, mutant *in vitro* expression has been performed (Akaboshi et al., 2001). Mutant alleles were produced by site-directed mutagenesis, the corresponding cDNAs cloned in expression vectors and the expression measured after transfection. Up to now more than 50 mutations abolishing or drastically reducing the enzyme activity to less than 5% have been found, except one producing an enzyme activity of 17% (Akaboshi et al., 2003).

Severity of the phenotype does not correlate with SSADH activity levels; in fact, even between cases with enzyme activity less than 5%, clinical features of patients were very different. This finding suggests that other modifying factors may be important in disease pathology, like polymorphisms in other metabolic enzymes affecting GABA metabolism, or altered expression of GABA and GHB receptors (Mehta et al., 2002).

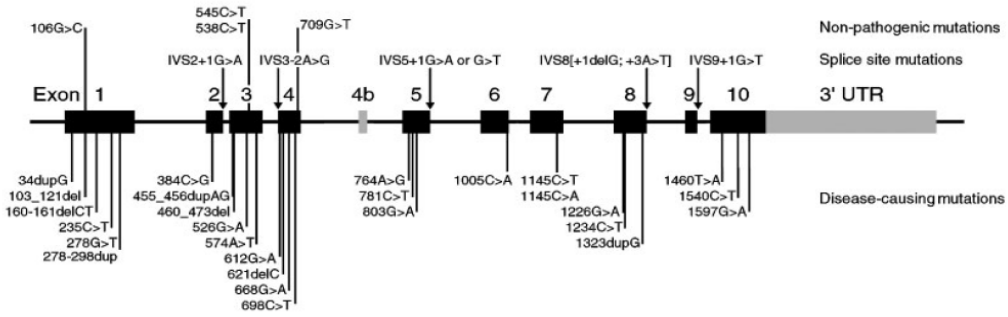

Fig. 6. *Mutations and polymorphisms in SSADH gene (Akaboshi et al., 2003).*

Non-pathological variability of SSADH gene

By sequencing the *SSADH* coding region in relatives of patients affected by 4-hydroxybutyric aciduria and in other families of European descent, normal nucleotidic variability was also explored. Many missense and samesense variants at polymorphic frequency were found (Blasi et al., 2002; Akaboshi et al., 2003; Blasi et al., 2006) (**Fig. 6**).

It is very interesting to note that most of the observed polymorphisms correspond to aminoacid changes. To investigate the functional consequences of polymorphic variations in coding sequence, in vitro expression studies were performed. The analysis displayed a wide variability of SSADH activities associated with different alleles, ranging from 47.6 to 86.7% as compared to the most common typel (Blasi et al., 2002). Thus, the level of polymorphism found in the coding region of the gene is large enough to predict inter-individual variation in enzyme activity.

The most common coding variant in *SSADH* was found to be a C>T transition in exon 3, at position 538 of the cDNA, corresponding to position 47015 of the reference genomic sequence (accession N° AL031230), leading to a histidine- tyrosine substitution at aminoacid position 180 of the native polypeptide. *In vitro* studies showed that enzymatic activity of the Tyr_{180} protein is about 82% of the activity of the Hys_{180} protein. By sequence comparison with several non-human primates, allele 538T was identified as the ancestral state (Blasi et al., 2006). It is very interesting to note that

the derived allele C determines the replacement of a tyrosine residue conserved from rodents to apes with a histidine never observed in other eukaryotes; this has increased its frequencies and now represents the vast majority world-wide.

SSADH as a susceptibility gene for cognitive phenotypes

SSADH has been supposed to play a role in a variety of phenotypes. In this context it is very interesting the recent discovery that SSADH is involved in other metabolic pathways. In fact, although it is reported to have a narrow substrate specificity, it has been showed that it also catalyses the oxidation of 4 hydroxy-*trans*- nonenal (HNE) which derives from fatty acid metabolism (Murphy et al. 2003; Meyer et al., 2004). HNE is a major, neurotoxic product of lipid peroxidation whose levels are elevated in multiple neurodegenerative disorders, like Alzheimer's disease (AD) and Parkinson's disease (Picklo et al., 2002) as well as in cerebral ischemia (McKracken et al., 2001). HNE alters cellular signaling and is cytotoxic through alkylation chemistry (Keller, 1997); its application *in vitro* can mimic pathological changes noted in AD, including inhibition of glutamate transporter and microtubular disruption (Murphy et al., 2003). Furthermore, *SSADH* gene has given linkage signals in families and association signals with a variety of disorders.

SSADH gene maps in 6p22, a chromosomal region showed to be associated with dyslexia. Dyslexia or Reading Disability (RD) is a mild hereditary neurological disorder that manifests as a persistent difficulty in learning to read and phonology, in children with otherwise normal intellectual functioning and educational opportunities. Other than an excellent positional candidate, *SSADH* gene represents a functional candidate for dyslexia because the delay in development of speech and language is one of the features of 4-HBA patients. There are conflicting reports on the fine mapping of a locus for dyslexia, but they all point to genes very close to *SSADH* on both sides, and there are also positive TDT tests for *SSADH* SNPs (Ahn et al., 2001; Londin et al. 2003; Cope et al., 2005; Deffenbacker et al., 2004).

SSADH has also been called in pathological phenotypes physiologically associated with an altered GABA metabolism, including paranoid schizophrenia (Zhang et al., 2005), juvenile myoclonic epilepsy and photosensitivity (Lorenz et al., 2006) and

neuroleptic malignant syndrome (Neu et al., 2002), and association studies have been performed in order to investigate the possible involvement of the gene in these pathologies.

Recently a role of SSADH has been supposed in the generation of oxidative stress, because it was shown that GHB, the compound accumulating in enzyme deficiency, does induce oxidative stress in cerebral cortex of rats.

All these observations underline the complexity and the multiple ramifications of the metabolic pathways of SSADH, and they make it plausible that the normal variation in the gene might be associated with subclinical phenotypes perhaps cognitive or behavioural.

The most important suggestions that SSADH normal activity is relevant to cognitive abilities, come from the analysis of patients affected by 4-hydroxybutyric aciduria. The degree of impairment resulting from SSADH deficiency is highly variable, ranging from serious neurological impairment to mild developmental delay. These patients invariably show various degrees of neurological symptoms like mental and psychomotor retardation, speech and language defects. Carrier parents, having a reduced SSADH activity, have also been reported to show EEG abnormalities (Dervent et al., 2004).

It is known that the increase of GABA and GHB concentrations in 4-HBA patients is due to the metabolic block caused by the anomalous SSADH enzyme, which has a very low activity, less than 5% of wild type in most of the cases.

As drastic reductions of SSADH activity led to accumulating of GABA and GHB causing the pathology, it is likely that common variants for the coding sequence determining a reduced but non-pathological enzyme activity, may influence endogenous levels of these metabolites and eventually contribute significantly to other phenotypes correlated to its metabolic pathway. In fact, 5-fold variations of SSADH activity in the general population have been reported (Gibson et al., 1991) and *SSADH* gene polymorphisms can cause these inter-individual variations of enzyme activity, as suggested by the experiments of *in vitro* expression of missense variants of the gene (see above).

Indeed, a possible involvement of SSADH in higher cognitive functions has been recently suggested by Plomin and co-workers, who showed an association of the functional C538T polymorphism with cognitive ability (Plomin et al., 2004). The study was carried out using two different designs, a case-control study and a family analysis.

Both experimental designs showed significant evidences of association between the functional polymorphism and the intelligence in the general population. In particular they showed that the less common allele (corresponding to lower activity enzyme variant) was significantly less frequent in high-IQ cases and was significantly less frequently transmitted by parents to high-IQ subjects as compared to null expectation.

The above observations also suggest that normal variability of *SSADH* gene may not be evolutionarily neutral.

Evolutionary implications of SSADH variability

Several studies are focused on the identification of genes that have been subjected to directional selection and could potentially contribute to the evolutionary process in humans. These studies analyze interspecific divergence comparing genes at the nucleotide level. Signatures of natural selection in human genome has been highlighted for many genes (Bamshad and Wooding, 2003, Sabeti et al., 2006).

In particular, many studies have linked specific genes to the evolution of human brain, a primary process during primate and particularly human evolution. One of this is *Microcephalin*, a regulator of brain size for which signatures of strong positive selection have been shown (Evans et al., 2004). Furthermore, Enard and co-worker (Enard et al., 2002), suggested that a variant of the highly conserved FOXP2 gene was driven to fixation by positive selection associated with the development of the human spoken language. This study supported the hypothesis that novel gene variants arisen after the divergence from the last common ancestor with chimpanzee gave rise to human specific traits (Dennis 2005).

The sensitive role of *SSADH* in cognitive capabilities triggered the investigation of *SSADH* from an evolutionary point of view.

It was already known that SSADH is a protein very conserved throughout evolution (Chambliss et al., 1995). Recently, in a work aimed at searching for possible evolutionary trends in the pattern of aminoacid substitutions, *SSADH* was sequenced in several primates (Blasi et al., 2006). A small number of substitutions was encountered and interspecific comparisons showed the signature of strong conservation. Furthermore, the constancy of nonsynonymous (Dn) versus synonymous (Ds) rates of nucleotide changes was tested, and it was found that the Dn/Ds ratio varies quite a lot

across primates branches. Nevertheless, on a background of strong conservation, the human specific branch showed a three time increase of the the Dn/Ds ratio, perhaps indicating a relaxed selection with an accelerated rate of aminoacid sobstitutions. Noteworth, the intraspecific variation in humans was found to accounts for a further increase of the Dn/Ds ratio. In fact 4 out of the 5 variants found resulted to be non-synonymous changes and only one synonymous; this distribution represents a pattern opposite to what seems the rule in primate phylogeny where most of the substitutions are non-synonymous. In fact in pairwise comparisons with human, in all species except the Bonobo an excess of synonymous changes is observed.

In summary this work shows that *SSADH* has undergone a strong conservation in primates and before primates, but in humans it shows a polymorphism with many aminoacid replacements; these data are compatible with a relaxed selection with an accelerated rate of aminoacid substitutions in the human lineage.

Aim of the work

During my PhD appointment I was involved in the study of different aspects of the phenotypic effects of the functional C538T polymorphism in the exon 3 of *SSADH*. In particular I focused my work on i) the effect of this polymorphism on the preservation of cognitive performance and on survival in the elderly; ii) the effect of natural selection on the global distribution of this polymorphism.

The preservation of cognitive ability is an important element in successful aging and one of the most important component of the quality of life in the elderly. It is known that the variability of cognitive functions can be due to both genetics and environmental factors, but genetic factors become more important with age, and account for at least 50% of the variance in cognitive function in elderly. The variability of *SSADH* may be involved in the conservation of cognitive function both because of its effect on cognitive abilities and for the suggested role in affecting oxidative stress. To verify this hypothesis we studied the association between the C538T polymorphism and preservation of cognitive function in the elderly in a sample population from southern Italy. In addition, because of the crucial role of cognitive conservation for survival chance in the elderly we also studied the correlation between C538T polymorphism and survival in the same population.

The global distribution of polymorphisms is extremely useful, in conjunction with the analysis of the flanking regions in human populations and human/primates sequences alignment, for the identification of alleles that have been favoured by natural selection. Although data have accumulated on the diversity of the regions flanking the C538T polymorphism and on the human/primates diversity, the world distribution of the two alleles of this polymorphism had not been systematically analyzed. In order to find out if natural selection had an effect in shaping the distribution of *SSADH* C538T alleles we analysed the world distribution of the two alleles.

2. The *SSADH* C538T polymorphism: relationship to cognitive functioning and to survival in the elderly.

Ofelia Leone, Francesco De Rango, et al., (2007). Submitted to Neurobiology of aging.

Abstract

The variability of the Succinic Semialdehyde dehydrogenase (*SSADH*) gene has been proved to affect both pathological and normal phenotypes correlated to cognitive function. We tested the association between the functional polymorphism C538T of the *SSADH* gene and the preservation of cognitive function in the elderly and its possible effects on survival. A sample of 514 subjects from southern Italy (age range 18-107 years) was screened for *SSADH* C538T variability. We found that within age range of 65-85 years the TT genotype was overrepresented in subjects with impaired cognitive function (MMSE≤ 23) compared to those with conserved cognitive function (MMSE>23). As the enzymatic activity of the protein encoded by allele T is 82.5 % of the activity of the protein encoded by allele C, our results suggest that the efficiency of the SSADH enzyme is important for the preservation of cognitive function in the elderly. In addition, we found that the TT genotype (which has a frequency of 5% in young adults) affects survival after the 65 years of age, and it becomes completely absent from our sample after the 80 years of age.

1. Introduction

SSADH (Succinic Semialdehyde Dehydrogenase) is a mitochondrial NAD(+)-dependent enzyme which is involved in the catabolism of Gamma-Amino Butyric Acid (GABA). SSADH deficiency (MIM 271980) is a rare recessive metabolic disorder, which leads to the neurotoxic accumulation of GABA and Gamma-Hydroxy Butyric Acid (GHB), thus resulting in 4-hydroxybutyric aciduria (Gibson, 2005; Pearl et al., 2005). SSADH deficiency, the most common disorder of GABA metabolism, is characterized by a wide spectrum of mild to very severe clinical features, including psychomotor and language retardation, hypotonia, seizures and ataxia (Pearl et al., 2003a). The large phenotypic variability observed in different families in relationship to the mutational spectrum of the *SSADH* gene suggested that modifying factors are of great importance in the disease pathology and that common variants of the gene may influence a broader spectrum of

cognitive ability in the general population (Akaboshi et al., 2003; Plomin et al., 2004). In this context, the C538T polymorphism (c.538, rs2760118, exon 3, www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2760118) appeared to be very interesting for different reasons. Firstly, the enzymatic activity of the protein encoded by the less frequent allele T (Tyr_{180}), is 82.5% of the activity of the His_{180} protein encoded by the more common C allele (Blasi et al., 2002). Secondly, by both case-control and parent-offspring analyses the C allele resulted to be associated with increased cognitive ability, thus showing that higher SSADH activity is associated with higher cognitive performance across the general population (Plomin et al., 2004). Moreover, a polymorphic DNA marker closely linked to *SSADH* C538T, exhibited significant departures from Mendelian trasmission in families of children with dyslexia (Deffenbacher et al., 2004). Finally, SSADH has been found to be involved in the detoxification of 4-hydroxy-2-nonenal (HNE), a neurotoxic aldehyde product of lipid peroxidation whose concentration is elevated in Alzheimer's and Parkinson's diseases (Murphy et al., 2003; Meyer et al., 2004). On the whole, these findings suggest that the *SSADH* C538T polymorphism contributes to the individual performance in cognitive ability at young age. We then wanted to test the hypothesis that the *SSADH* C538T polymorphism also plays a role in the variability of the cognitive decline which characterizes elderly people after age 65 (Grigoletto et al., 1999; De Ronchi et al., 2005). In fact, a low efficiency of the SSADH enzyme may induce oxidative stress either by inefficient aldehyde(s) clearance or through increased concentrations of GHB in tissues and organs (Sgaravatti et al., 2007). Therefore, if the *SSADH* C538T polymorphism contributes to cognitive ability at young age, such a contribution should be enhanced in the elderly, in view of the oxidative stress status which characterizes the aging process (Longo and Fabrizio, 2002). Should this be the case, this polymorphism might have important consequences on the quality of aging and on life span, as "cognitive functioning is central in successful aging and longevity" (Bathum et al., 2007 and references therein). The aim of the present work was to verify the association of the functional polymorphism *SSADH* C538T with the preservation of cognitive function and survival in the elderly. We analyzed the distribution of *SSADH* C538T genotypes in two subgroups of 65-80 year old subjects with different scores in the MMSE test (≤ 23;> 23). It is worth noticing that the prevalence of cognitive impairment has been found to increase with age among persons over 65 years but it seem to reach a plateau in people aged 85 years and older (De Ronchi et al. 2005 and references therein). Furthermore, we tested the possible effect on survival of the polymorphism C538T of the *SSADH* gene, by

applying the genetic-demographic approach to determine the empirical survival function of old subjects carrying different genotypes at *SSADH* C538T locus.

2. Materials and Methods

2.1. Samples

The samples analyzed in the present study (514 subjects, age range 18-107) were recruited in different campaigns. The subjects aged between 65 and 107 years were recruited in the context of the ECHA project (European Challenge for Healthy Ageing, www.biologia.unical.it/echa). The recruitment campaign took place in Calabria (southern Italy) in the period January 2002- December 2004. The subjects, born and living in Calabria, and whose parents and grandparents were native of the same area, were identified from the general registry and then invited to take part in the study. For each subject who complied with the request, his/her origin in Calabria was verified in the birth-registers of the Municipality Office where the subject was born. Routine blood analyses were carried out and a complete clinical and geriatric assessment, comprising cognitive measurement, was performed on these subjects.

The younger sample, aged between 18 and 64, was recruited in the frame of blood donation campaigns, carried out in the Calabrian region. The origin of each individual in the specific geographic area up to the maternal grandmother was ascertained by interview. All the subjects included in the study were free of clinically manifested pathologies, and gave their informed consent to participate.

2.2.DNA analysis

DNA was extracted from venous blood by standard techniques. For genotyping the C538T polymorphism (rs2760118, see web reference) we used the following method. First we amplified the *SSADH* exon 3 using the primers S13 (5'CCCCACTCTATGGGTATCG3') and S32 (5'CTCCCACCCTAGGATCTTG3') under the following conditions: 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 56°C for 30 sec, and 72°C for 40 sec, with a final elongation step at 72°C for 5 min. The PCR reaction mix contained 0.20 mM dNTPs, 1U Taq polymerase and 1 mM $MgCl_2$. One μl of the exon 3 amplification product was used to prime an allelic specific, nested PCR of 10 cycles using the same profile described above. The nested PCR was carried out using the S32 primer and the allele specific primers SSADH538-1 (5'HEX-GTGTTTACGGAGACATTATCC-3') and SSADH538-2 (5'FAM-

GTGTTTACGGAGACATTATCT-3'). The reaction mix contained 0.20 mM dNTPs, 0.5U Taq polymerase and 1.5 mM $MgCl_2$. One μl of the reaction product was mixed with 12 μl of formamide, loaded on an ABI 310 automated sequencer and analysed with the GeneScan software, with Tamra as internal standard. The use of allele specific primers marked with different fluorochromes allows the identification of the two alleles by differently coloured peaks.

2.3.Cognitive assessment

Cognitive function was assessed by the Mini Mental State Examination (MMSE) (Folstein et al. 1975). Since the test is affected by age and school-attendance, the scores were corrected for these factors according to Magni et al. (1996).

A score of 23 was considered as a cut-off for recognizing impaired (MMSE equal to or lower than 23) or conserved cognitive function (MMSE higher than 23) (Rait et al., 2005; Anderson et al., 2007).

2.4.Statistical Analysis

The random assortment of the alleles in the entire sample (Hardy–Weinberg equilibrium), as well as in the sample categorized according to the MMSE scores, was tested by allele shuffling with 5000 random permutations.

Statistical comparisons between groups were performed by Likelihood Ratio test on SPSS 14.0 (SPSS Inc., Chicago, IL).

The survival function of subjects older or equal to 65 years, carriers and non carriers of the TT genotypes, was determined by applying a genetic-demographic approach (GD approach). The method allows the estimation of survival functions for candidate alleles and genotypes in cross-sectional samples of unrelated individuals. A detailed description of the analytical procedure is reported elsewhere (Dato et al., 2007). Briefly, the GD analysis is based on: i) a survival model; ii) a model that links gene frequencies and survival; iii) a procedure for estimating model parameters starting from demographic and genetic data. Let $S(x)$ be the survival function of the population at age x, defined as the fraction of individuals born at a certain time t_0 (N_0) and still alive at time $t_0 + x(N(x))$: $S(x) = N(x) / N(0)$; this function is computed by using death counts per calendar year and per year of birth, and cohort initial values as reported for the Italian population in the Human Mortality Database (www.mortality.org). $S_a(x)$ and $S_b(x)$ are the marginal survival functions evaluated at age x of carriers and non carriers, respectively, of an

allele/genotype; the relation between them and the survival function of the whole population is $S(x) = PS_a(x) + PS_b(x)$ where P is the initial (i.e., at birth) frequency of carriers in the population.

In order to take into account the changes in mortality experienced by all the cohorts considered in the study, a "synthetic" survival function of the general population was constructed. The marginal survival functions $S_a(x)$ and $S_b(x)$ of carriers and non carriers of genotypes were determined taking into account the survival function $S(x)$ of the whole population and the initial frequencies (i.e., at birth) frequency of carriers in the population, according to the relation $S(x) = PS_a(x) + PS_b(x)$.

The Matlab 6.1 package was used for all the above analyses.

3. Results

In order to explore the influence of the *SSADH* C538T polymorphism on the preservation of cognitive functioning, we analyzed the correlation between *SSADH* genotypes and MMSE scores.

Genotypic and allelic frequencies at the *SSADH* C538T polymorphism were determined in the whole sample of 514 individuals (CC=323; CT=175; TT=16; p = 0.151 at HWE equilibrium).

The association analysis was carried out in the subsample with an age range of 65-85 (N= 155). We used MMSE scores to define two phenotypic groups as described in Methods. Table 1 reports the results obtained. Very interestingly, the genotype distribution (CC, CT, TT) differed between the groups categorized according to MMSE scores (p=0.05). In particular, the observed frequency of the TT genotype in the sample of individuals having MMSE scores lower than 23 was about twice than expected (6 *vs.* 3.26) and it was significantly higher than that found in the sample having MMSE scores higher than 23 (p=0.020). In fact, in the group of subjects having a preserved cognitive function, the TT genotype is rather rare (1%) and lower than expected under the assumption of independence between polymorphism and phenotype (1 *vs.* 2.79). These results suggested that *SSADH* C538T polymorphism contributes to cognitive ability in the elderly; considering also that cognitive functioning is central in successful aging and longevity, we explored the association between the polymorphism and survival.

We carried out a genetic-demographic analysis on subjects aged between 65 and 107, categorized for *SSADH* C538T locus. The initial frequency of carriers of different

genotypes in the population (parameter P in the genetic-demographic method, see Materials and Methods) was calculated on a subsample of 250 subjects, aged between 18 and 64 years (CC= 149; CT=92; TT=9; p =0.323 at HWE equilibrium). The application of the GD method implies the assumption of dominance of one over another. On the basis of the functional effects of C and T alleles, we assumed the C>T dominance relationship. Fig.1 shows the maximum likelihood estimates of the survival functions in TT subjects (blue curve) and CC *plus* CT subjects (red curve), compared to the general population for subjects aged between 65 and 107. The survival function of the TT homozygous subjects is lower with respect to CC *plus* CT subjects at all ages. Furthermore, the TT genotype showed a dramatic reduction of its distribution at 80 years and was completely absent in our sample after this age.

Discussion

The SSADH C538T polymorphism contributes to individual cognitive performance in young subjects, possibly because of the reduced activity of the Tyr_{180} form of the enzyme (corresponding to the T allele) with respect to the His_{180} protein (corresponding to the C allele) (Plomin et al., 2004). Considering the effects of a low efficiency of the SSADH enzyme in inducing oxidative stress, the role of stress in promoting the aging process, together with the importance of cognitive functioning in successful aging and longevity, the *SSADH* C538T polymorphism can contribute to cognitive ability also in the elderly. The analysis of subjects ranging from 65-80 years, categorized according their cognitive function, demonstrated that the TT homozygous genotype is significantly over-represented in subjects with impaired cognitive function (MMSE score $\leq$ 23) with respect to the group of subjects with conserved cognitive function (MMSE >23) (p=0.020).

Thus, our results consistently suggest that the SSADH C538T polymorphism is correlated with cognitive function in the elderly, and that the TT genotype can be considered a "frailty phenotype" (Bathum et al. 2006) which increases the susceptibility to cognitive impairment. The latter has important consequences on survival chances as can be expected considering the high correlation between cognitive functioning and survival.

In fact, the analysis of the survival functions in the same sample analyzed for the association between the *SSADH* variability and cognitive function (people aged between 65 and 107 years) demonstrated as the survival of TT homozygous subjects is always lower with respect to CC *plus* CT subjects, at all ages. Moreover, the analysis showed as

TT genotype dramatically decreases at old ages and it is completely absent after the 80 years of age.

In order to confirm the association between the C538T polymorphism and preservation of cognitive function in the elderly, the study will be replicated using a larger sample for which a new recruitment campaign is presently underway. However, our current data are encouraging and, providing the results are confirmed, medical strategies aimed at preventing cognitive deterioration in TT carriers may be designed. Taking into account the dramatic increase in numbers of elderly people in industrialized countries, this may contribute to improve the quality of the life in a consistent segment of population.

Genotypes	MMSE≤23 Abs	MMSE≤23 Rel ±SE	MMSE>23 Abs	MMSE>23 Rel ± SE
CC	45 (42.261)	0.652 ± 0.057	56 (57.794)	0.651 ± 0.051
CT	18 (23.478)	0.261 ± 0.053	29 (25.413)	0.337 ± 0.051
TT	6 (3.261)	0.087 ± 0.034	1 (2.794)	0.012 ± 0.012
Total	69		86	
Heterogeneity test		p = 0.05		

Table 1. Absolute (Abs) and Relative (Rel) genotype frequencies of the *SSADH* C538T polymorphism in the subsample with age range 65-85 years categorized according to MMSE score. The genotypic counts expected under the hypothesis of random allele combination is given in parenthesis. SE: Standard Error.

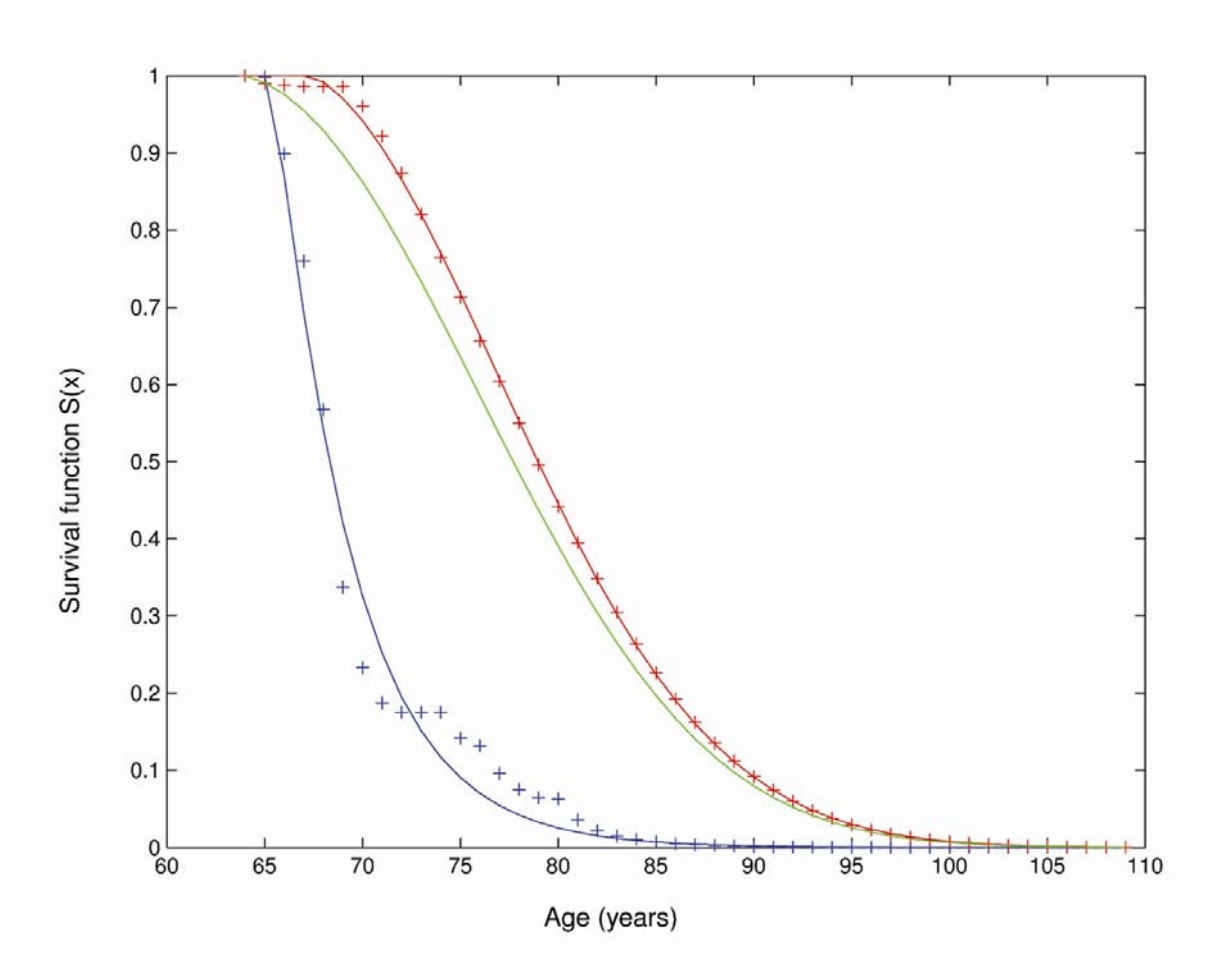

Fig. 1. Maximum likelihood estimates of the survival functions of TT homozygous subjects ($S_a(x)$; blue curve) and CC *plus* CT subjects ($S_b(x)$; red curve) at the *SSADH* C538T locus, aged between 65 and 107 years. The synthetic survival function of the Italian population (Ss(x); green curve), is also reported as a reference.

3. A human derived SSADH coding variant is replacing the ancestral allele shared with primates

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ORIGINAL ARTICLE

A human derived SSADH coding variant is replacing the ancestral allele shared with primates

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Abstract

Background: A growing number of reports describe markers with high frequencies of the ancestral alleles in Africa, contrasting with high frequencies and possibly fixation of derived variants out of Africa. Such a pattern can be explained by either neutral or non-neutral processes.

Aim: The study examined worldwide frequencies of two non-synonymous variants in NAD^+-dependent succinic semialdehyde dehydrogenase (SSADH), in a search for possible signatures of natural selection favouring the derived alleles.

Subjects and methods: The typing of 1574 subjects were compiled, representing 60 populations from all continents. SSADH haplotype frequencies were correlated across 52 populations to those of 260 single nucleotide polymorphism (SNP) markers deposited in the CEPH database and of markers reported to be under positive Darwinian selection.

Results: In the world population, the c.538C variant is proceeding to replace the ancestral c.538T, shared with primates. The overall population differentiation is within the normal range. A significant correlation was also found between the frequencies of the derived alleles in SSADH and Microcephalin (MCPH1), which showed concerted changes worldwide and, at least in Asian populations, also on a restricted geographical scale.

Conclusion: The analysis of robust correlations based on a large panel of populations is potentially able to identify clusters of genomic regions or genes showing co-evolution of the frequencies of derived alleles.

Keywords: *SSADH, Darwinian selection, human population, co-evolution*

Introduction

The identification of alleles that have been subjected to positive natural selection is one of the challenges in human biology. The best examples derive from candidate genes for which

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there are prior hypotheses of selection. These studies combined the analysis of interspecific divergence, molecular diversity associated with different allelic variants and polymorphism in human populations.

In the present study, we focus on the polymorphic variation of a key enzyme in γ-aminobutyric acid (GABA) metabolism, i.e. mitochondrial NAD^+-dependent succinic semialdehyde dehydrogenase (SSADH; ALDH5A1, EC 1.2.1.24). SSADH catalyses the oxidation of succinate semialdehyde to succinate, which is the final catabolite of the GABA shunt. In addition, it has been demonstrated that in rat central nervous system SSADH is also responsible for the oxidation of 4-hydroxy-2-nonenal, a cytotoxic product of lipid peroxidation (Murphy et al. 2003). We mapped human SSADH in 6p22.2-p22.3 as a single copy gene (Malaspina et al. 1996) and characterized its genomic and coding structures. The gene consists of 10 exons encompassing over 38 kb. The complete open reading frame (ORF) is 1605 bp (acc. no. Y11192) encoding for 535 amino acids, with the first 47 residues recognized as mitochondrial targeting peptide (Chambliss et al. 1998).

Re-sequencing of the SSADH coding region in relatives of SSADH-deficient patients (Akaboshi et al. 2003) and in other families of European descent (Blasi et al. 2002) revealed the presence of several missense and samesense variants at polymorphic frequencies among which two are common, i.e. c.545 C > T and c.538 T > C. In particular, the latter causes the replacement of a tyrosine residue conserved in primates and rodents with a histidine never observed in other eukaryotes. We hypothesized that the high frequencies of c.538C are the result of positive selection (Blasi et al. 2006).

Here we report on the allele frequencies at positions c.538 and c.545 in 60 population samples representative of all continents. Moreover, we exploited a large number of single nucleotide polymorphisms (SNP) markers typed in the same set of populations to test for parallel changes of frequencies with other positively selected markers.

Materials and methods

Subjects

The complete dataset consists of the Human Genome Diversity Project (HGDP) Panel (Cann et al. 2002) with 147 subjects already reported by Blasi et al. (2006); 218 newly typed Italian, UK and Nigerian subjects; and 101 subjects from six populations reported here for the first time (Table I). The total amounts to 1574 individuals representing 60 populations from the five continents. DNA was extracted from venous blood in EDTA, blood drops adsorbed on paper or buccal smear. Informed consent was obtained verbally in all cases.

Methods

Individual genotypes at positions c.538 (rs2760118) and c.545 (rs3765310) were determined with two independent methods. In the first one, SSADH exon 3 was amplified with specific primers (Blasi et al. 2002), spotted on nylon membranes and hybridized with 32-P labelled ASO probes as described (Blasi et al. 2006).

In the second method, 1 μL of the exon 3 amplification product was used to prime each of two nested PCR of 10 cycles (94°C, 1 min; 56°C, 30 s; 72°C, 40 s; after a predenaturation at 94°C, 4 min), with the primers S13 (Blasi et al. 2002), SSADH538-1

Table I. Haplotype frequencies in the 60 population samples.

Continent	Geographic origin	Population	Sample origin	Sample size	Haplotype		
					C–C	T–C	T–T
Africa	Algeria	Mozabite	HGDP panel	30	0.583	0.334	0.083
	Senegal	Mandenka	HGDP panel	17	0.471	0.500	0.029
	Nigeria	Yoruba	HGDP panel	24	0.563	0.438	–
	Nigeria	Mixed	Blasi et al. (2006) and this paper	34	0.603	0.382	0.015
	Central African Republic	Biaka Pygmies	HGDP panel	20	0.600	0.350	0.050
	DR Congo	Mbuti Pygmies	HGDP panel	14	0.357	0.643	–
	Kenia	Bantu NE	HGDP panel	12	0.792	0.208	–
	Namibia	San	HGDP panel	7	0.429	0.571	–
	South Africa	Bantu	HGDP panel	8	0.437	0.438	0.125
Europe	France	French Basque	HGDP panel	24	0.750	0.250	–
	France	French	HGDP panel	29	0.707	0.259	0.034
	Italy	Sardinian	HGDP panel	28	0.839	0.143	0.018
	Italy	Bergamo	HGDP panel	13	0.846	0.115	0.038
	Italy	Tuscan	HGDP panel	8	0.624	0.313	0.062
	Italy	Southern Italian	Blasi et al. (2006) and this paper	317	0.778	0.174	0.049
	UK	English	Blasi et al. (2006) and this paper	22	0.636	0.273	0.090
	Orkney Islands	Orcadian	HGDP panel	14	0.750	0.250	–
	Russia	Russian	HGDP panel	25	0.520	0.400	0.080
	Russia	Mordvin-Erzya	this paper	19	0.763	0.211	0.026
	Russia	Perm Russian	this paper	18	0.806	0.167	0.028
	Russia	Adygei	HGDP panel	17	0.765	0.235	–
Asia	Israel	Bedouin	HGDP panel	49	0.684	0.245	0.071
	Israel	Drusi	HGDP panel	48	0.708	0.188	0.104
	Israel	Palestinian	HGDP panel	50	0.810	0.130	0.060
	Pakistan	Brahui	HGDP panel	25	0.700	0.260	0.040
	Pakistan	Balochi	HGDP panel	24	0.792	0.188	0.021
	Pakistan	Hazara	HGDP panel	25	0.840	0.160	–

(Continued)

Table I. Continued.

Continent	Geographic origin	Population	Sample origin	Sample size	Haplotype C–C	Haplotype T–C	Haplotype T–T
	Pakistan	Makrani	HGDP panel	25	0.559	0.321	0.119
	Pakistan	Sindhi	HGDP panel	25	0.700	0.200	0.100
	Pakistan	Pathan	HGDP panel	24	0.583	0.396	0.021
	Pakistan	Kalash	HGDP panel	25	0.740	0.200	0.060
	Pakistan	Burusho	HGDP panel	25	0.760	0.120	0.120
	China	Tuja	HGDP panel	10	0.850	0.100	0.050
	China	Yizu	HGDP panel	10	0.850	0.050	0.100
	China	Miazou	HGDP panel	10	0.800	0.200	–
	China	Orogen	HGDP panel	9	0.833	0.112	0.055
	China	Daur	HGDP panel	10	0.750	0.250	–
	China	Mongol	HGDP panel	10	0.750	0.200	0.050
	China	Hezhen	HGDP panel	10	0.800	0.100	0.100
	China	Xibo	HGDP panel	9	0.880	0.056	0.055
	China	Uighur	HGDP panel	10	0.950	–	0.050
	China	Dai	HGDP panel	10	0.698	0.052	0.248
	China	Han	HGDP panel	44	0.863	0.091	0.045
	China	Lau	HGDP panel	10	1.000	–	–
	China	She	HGDP panel	10	0.849	0.101	0.049
	China	Tu	HGDP panel	9	0.944	–	0.056
	Japan	Japanese	HGDP panel	31	0.710	0.129	0.161
	Cambodian	Cambodian	HGDP panel	11	0.864	0.136	–
	Siberia	Mansi	this paper	7	0.714	0.286	–
	Siberia	Khanti	this paper	16	0.719	0.250	0.031
	Siberia	Yakut	this paper	24	0.750	0.125	0.125
	Siberia	Yakut	HGDP panel	25	0.679	0.161	0.159
	Siberia	Chukcee	this paper	17	0.882	0.088	0.029
Oceania	New Guinea	Papuan	HGDP panel	17	0.469	0.148	0.381
	Bougainville	NAN Melanesian	HGDP panel	22	0.432	0.023	0.545
America	Mexico	Pima	HGDP panel	25	0.920	0.080	–
	Mexico	Maya	HGDP panel	25	0.900	0.100	–
	Colombia	Colombian	HGDP panel	13	0.769	0.231	–
	Brazil	Karitiana	HGDP panel	24	0.625	0.375	–
	Brazil	Surui	HGDP panel	21	0.762	0.238	–

(5′HEX-GTGTTTACGGAGACATTATCC-3′) and SSADH538-2 (5′FAM-GTGTTT ACGGAGACATTATCT-3′) or primers S32, SSADH545-1 (5′HEX-CCCGCCTGTCCT TTGCCG-3′) and SSADH545-2 (5′FAM-CCCGCCTGTCCTTTGCCA-3′). Each reaction contained 0.20 mM dNTPs, 0.5 U Taq polymerase and 1.5 mM or 1 mM $MgCl_2$ for positions c.538 and c.545, respectively. A 1 μl volume of the product of each reaction was mixed with 12 μL of formamide, loaded on an ABI 310 automated sequencer and analysed with the GeneScan software, with Tamra as internal standard. Allele states were identified by black or blue peaks of 178 and 191 bp for c.538 and c.545, respectively. Reference samples typed with either method were used as internal standards for the other method.

Data analysis

Haplotypic reconstruction of SNPs genotyping data were obtained with the program PHASE2 (Stephens et al. 2001) and further analysed with Arlequin 3.01 (Excoffier et al. 2005). The Hardy–Weinberg equilibrium at haplotypic level was tested by Markov chain with 100 000 steps. Significance of diversity indexes was obtained by comparison with null distributions obtained with 5000 permutations.

Allele frequencies for 260 SNP markers were determined from the original genotype data obtained in HGDP. Briefly, the Centre d'Etude du Polymorphisme Humain (CEPH) database (http://www.cephb.fr/hgdp-cephdb/main.php), as of April 2006, was queried. For each chromosome biallelic markers were selected, and dumping for all populations was requested in Arlequin format. The corresponding .arp file was further edited, imported into SPSS and analysed.

Correlations with geography were computed by (a) giving positive and negative values to Northern and Southern latitudes, respectively, to represent the overall latitudinal shift from the southernmost to the northernmost location and (b) using absolute Northern and Southern latitudes to represent variables that vary symmetrically around the equator, such as daylight variation.

Spatial autocorrelation of the frequencies of haplotype C–C was performed with the program SAAP (Sokal and Oden 1978) by using spherical distances. Only African, European and Asian samples were included, in order to avoid comparisons between pairs of populations separated by large bodies of water. In a first run, pairwise inter-population distances were grouped into 10 classes of equal width, each ranging 1.516 km. In a second run, 10 classes of 1000 km plus a final class ranging 10 000–15 200 km were used.

Results

The two polymorphic positions analysed in this work lie only 7 bp apart. By comparison with several non-human primates, we had already determined that the ancestral states at the two positions are c.538T and c.545C, respectively (Blasi et al. 2006).

Of the possible nine combined genotypes, only six were actually found. Haplotypic reconstruction produced unequivocal results in all populations, with only three inferred haplotypes and complete linkage disequilibrium between the two SNPs. All population samples turned out to be in Hardy–Weinberg equilibrium at haplotypic level, with the exception of the Yakut sample ($p = 0.048$) not included in the HGDP panel.

Table II. Apportionment of diversity by analysis of molecular variance.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among continents	4	25.434	0.01131	5.29
Among populations within continents	55	23.395	0.00521	2.44
Within populations	2928	577.448	0.19722	92.27
Total	2987	626.277	0.21374	
Fixation indices				
Fsc: 0.02575				
Fst: 0.07732				
Fct: 0.05293				

Haplotypic frequencies are reported in Table I. By virtue of the complete association, the frequencies of haplotypes C–C and T–T equal the frequencies of c.538C and c.545T, respectively.

A clear geographic pattern can be observed in the old world. Haplotype C–C is less common in Africa. Its frequencies in western sub-Saharan Africa are never above 0.6 and, correspondingly, the highest frequencies of the ancestral haplotype T–C are observed here. In Europe, the frequency of C–C is always above 0.6. In Asia, with the exception of Pakistani Makrani and Pathan, the frequency of haplotype C–C is always above 0.7. In Central and Southern America the frequencies of haplotype C–C vary between 0.6 and 0.9, and haplotype T–T is not observed. Finally, Papua New Guinea and Melanesia display unusually low and high frequencies of haplotypes C–C and T–T, respectively.

The two replicate samples of Nigerians and Yakut produced very similar results. Conversely, similar frequencies were observed across multiple Russian samples collected, in poor agreement (pairwise Fst = 0.07–0.11; $p = 0.03$–0.01) with the single Russian sample represented in HGDP panel.

We used haplotypic frequencies to assay the degree of inter-population divergence by analysis of molecular variance (AMOVA). The results (Table II) show that most of the variance is within populations, with an overall Fst of 0.077. Analysis of the residual variation shows a greater divergence among continents (Fct = 0.0529, $P < 10^{-4}$) than within continents (Fsc = 0.0257, $P < 10^{-4}$). When sub-Saharan African populations are compared with the remaining populations, Fct increases to 0.102 ($P < 10^{-4}$), in line with the geographic clustering of populations carrying haplotype T–C at high frequencies.

Spatial autocorrelation confirmed this pattern, with significantly positive Moran I values at distances lower than 3000 km (mainly intra-continental) and significantly negative values at distances larger than 9000 km (all inter-continental).

Haplotype C–C reveals a highly significant correlation with latitude ($r = 0.442$, 58 d.f., $p < 0.0001$) and, to a lesser extent, with distance from the equator ($r = 0.319$, $p < 0.02$). A world map displaying graphically the frequencies of this haplotype is available at http://www2.bio.uniroma2.it/biologia/laboratori/lab-geneticaumana/geneuma-pubb.htm

This geographical distribution has an evident similarity with that of microcephalin (MCPH1), a gene for which strong evidence in favour of directional selection has been recently put forward (Evans et al. 2005). In order to quantify the similarity of haplotype frequencies, we computed the linear correlation coefficient across the 52 population typed for both markers. The overall linear correlation coefficient between SSADH haplotype C–C and MCPH1 haplotype D is 0.578 (Table III, top left). To assay whether this similarity is merely coincidental or may reflect a common history of the two markers in different populations, we analysed it in the context of other empirical data. To this aim,

Table III. Linear correlation coefficients (r) between frequencies of SSADH haplotype C–C and MCPH1 haplotype D with and without controlling for geographical coordinates (d.f. and P in parentheses).

	Continent					
Correlation	All	Africa	Europe	Asia	Oceania	America
Raw	0.578 ($49, 10^{-4}$)	0.475 (6, NS)	−0.049 (6, NS)	0.374 (26, 0.05)	NA	−0.875 (3, NS)
Controlled for latitude, longitude	0.465 (47, 0.001)	0.410 (4, NS)	−0.478 (4, NS)	0.404 (24, 0.04)	NA	−0.336 (1, NS)

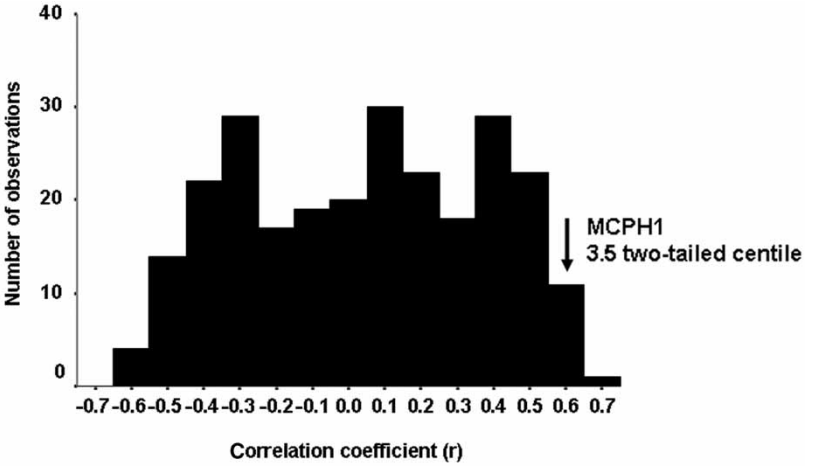

Figure 1. Histogram of linear correlation coefficients between the frequencies of SSADH haplotype C–C and those of alleles marked 1 in 260 SNPs of the CEPH database. Each column represents the number of markers which produced the indicated correlation coefficient in a pairwise comparison with SSADH haplotype C–C.

we considered all SNP markers reported in the CEPH database and tested in the same 52 populations. The histogram of linear correlation coefficients between the frequency of SSADH haplotype C–C and those of the allele marked 1 at each locus is shown in Figure 1. The distribution ranges between −0.597 and +0.660. This distribution identifies the observed correlation between SSADH and MCPH1 as unusually strong, in the 3.5 centile (two-tailed).

However we noticed that, due to the distribution of land masses, any inter-continental differentiation is likely to imply covariation with either latitude or longitude or both and, in turn, spurious correlations between markers. Thus we cannot rule out that the significant covariation between SSADH and MCPH1 is the result of purely demographic phenomena associated with the inter-continental dispersal of human populations. We then analysed the same correlation separately for each continent prior and after controlling for geographical covariates (Table III). The world correlation after controlling for latitude and longitude is still significant (Table III, bottom left). As expected, the correlation coefficients are less significant when determined within each continent. Nevertheless, in Asia, the continent represented with the largest number of samples, the correlation is still significant without control and is further increased upon controlling for latitude and longitude.

Discussion

SSADH c.538C > T and c.545C > T are two commonly occurring non-synonymous variants which alter the biochemical properties of a key enzyme of the GABA degradative pathway (Akaboshi et al. 2003). We previously identified c.538T and c.545C as the ancestral states at the two sites. Based on the pattern of diversity of linked markers, we deduced that haplotypes carrying c.538C have a shallow genealogy in Eurasia, one of the signatures of either a rapid population expansion or of positive Darwinian selection, or both. Here we explored a large panel of populations to obtain a more detailed view of the geographical pattern in the distribution of SSADH haplotypes.

The overall population differentiation is within the normal range (Akey et al. 2002). The common and widespread presence of the derived haplotype C–C in Africa is most likely explained by its origin before the exit of modern humans out of the continent. Nowadays this haplotype has reached high frequencies in Europe and near-fixation in Eastern Asian populations. In addition, the results in north-eastern Eurasians and all American samples denote that this haplotype had high frequencies in the ancestors of American populations. Its frequencies in the South American Karitiana and Surui may have been affected by the historical small effective sizes of these populations (Kidd et al. 1991; Kohlrausch et al. 2005).

As for haplotype T–T, it characterizes Oceanian populations and reaches frequencies above 0.10 only in Asian populations. We have shown (Blasi et al. 2006) that in Europeans this haplotype is associated with a third non-synonymous variant, c.106G > C (rs4646832). This variant is expected to cause a further reduction of the enzyme activity, as it alters the mitochondrial entry portion of the corresponding polypeptide. As this latter variant lies 8.3 kb upstream from c.538 on genomic DNA, the same association in non-European populations needs experimental investigation. If confirmed, further studies in Oceanian populations may help in identifying possible phenotypes associated with the standing SSADH variation.

In summary, as a whole, c.538C is proceeding to replace the ancestral c.538T, shared with primates.

A growing number of reports in the literature describe markers with a geographic pattern characterized by high frequencies of the ancestral alleles in Africa, contrasting with high frequencies and possibly fixation of derived variants out of Africa. Two main classes of hypotheses can be proposed for such a pattern in the frame of the out-of-Africa model for the dispersal of modern humans. The first class posits that the reduced effective size of populations involved in trans-continental migrations was associated with large stochastic fluctuations in allele frequencies which, in some cases, may have enriched Asia, Europe and later Oceania and America in the derived alleles. Modelling of the process of population growth during colonization is beginning to show the spectrum of geographical patterns expected for newly arisen mutations under this scenario (Klopfstein et al. 2006). The second, non-mutually exclusive class of hypotheses, considers positive natural selection in favour of the derived allele, possibly as a result of the exposure to new environments found out of Africa. Molecular data alone provide essential information to rule out the standard neutral model for a panmictic population, but can hardly discriminate between the two above hypotheses, due to the secondary demographic expansions associated with human dispersals. Therefore a precise description of the phenotypic effects of the variants under scrutiny is essential to identify the factor(s) potentially able to drive Darwinian selection (Thompson et al. 2004; Vander Molen et al. 2005).

We measured the enzyme activity of several SSADH alleles (Blasi et al. 2002; Akaboshi et al. 2003). The effects of alleles responsible for complete enzyme deficiency in altering the balance between GABA and its metabolites *in vivo* are well described in both patients and animal models (Gibson et al. 1998; Hogema et al. 2001). On the other hand, more subtle effects at the biochemical and/or subclinical level for the commonly occurring variants remain to be ascertained. Initial evidence that SSADH normal activity is relevant to cognitive abilities derives from the mental, psychomotor and language symptoms in patients affected by SSADH deficiency (OMIM 271980), as well as EEG abnormalities in their parents (Dervent et al. 2004). Indeed, a possible involvement of SSADH in higher cognitive functions has been suggested by Plomin et al. (2004), who showed that c.538C is significantly associated with higher performances.

In the absence of more compelling evidence on phenotypes associated with normal SSADH variation, we explored the covariation across human populations with another marker for which strong evidence of positive selection was produced, i.e. MCPH1. Although to a different extent, SSADH and MCPH1 share some evolutionary features: first, they both show accelerated rates of amino acid substitution in the human lineage as compared to apes (Evans et al. 2004; Wang and Su 2004); and second, in both genes a derived allele has increased in frequency and now represents the majority in human populations (Evans et al. 2005).

In contrast with the above gene-specific results, genome-wide scans have failed to detect the same signals. For SSADH the diagnostic polymorphism at c.538 was not included in the dataset of Bustamante et al. (2005). We also inspected the results by Voight et al. (2006) with the Haplotter software (http://hg-wen.uchicago.edu/selection/haplotter.htm) for signals of selection in the SSADH region. The indexes iHS, Fay and Wu's *H* and Tajima's *D* show no peaks of significance in a window of 5 Mb distally to SSADH. iHS and *D* show a single peak of marginal significance as far as 4.5 and 1.5 Mb proximally to SSADH, respectively. Large Fst values in the comparison between Yorubas and Asians are observed at SNPs located not closer than 600 kb proximally to SSADH. The fact that none of the SNPs here examined are included the Voight's dataset and that they have frequencies far from intermediate may explain the above findings. For MCPH1, the same reports detected weak negative selection or no signal, respectively.

The results presented here do not provide direct conclusive evidence for or against selection at c.538. However, the significance of correlation between the frequencies of the derived alleles in the two genes resisted statistical controls showing concerted changes worldwide and, at least in Asian populations, also on a restricted geographical scale. Conversely, positively selected alleles at other loci (Thompson et al. 2004; Mekel-Bobrov et al. 2005) did not reveal significant correlations (not shown).

The analysis of robust correlations based on a wide panel of populations is potentially able to identify enlarged clusters of genomic regions or genes showing this form of co-evolution. The finding of shared biological processes or molecular functions within a cluster may help in discriminating neutral versus non-neutral changes and would give important indications on the phenotypic features upon which selection may have acted.

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References

Akaboshi S, Hogema BM, Novelletto A, Malaspina P, Salomons GS, Maropoulos GD, Jakobs C, Grompe M, Gibson KM. 2003. Mutational spectrum of the succinate semialdehyde dehydrogenase (ALDH5A1) gene and functional analysis of 27 novel disease-causing mutations in patients with SSADH deficiency. Hum Mutat 22:442–450.

Akey JM, Zhang G, Zhang K, Jin L, Shriver MD. 2002. Interrogating a high-density SNP map for signatures of natural selection. Genome Res 12:1805–1814.

Blasi P, Boyl PP, Ledda M, Novelletto A, Gibson KM, Jakobs C, Hogema B, Akaboshi S, Loreni F, Malaspina P. 2002. Structure of human succinic semialdehyde dehydrogenase gene: Identification of promoter region and alternatively processed isoforms. Mol Genet Metab 76:348–362.

Blasi P, Palmerio F, Aiello A, Rocchi M, Malaspina P, Novelletto A. 2006. SSADH variation in primates: Intra- and interspecific data on a gene with a potential role in human cognitive functions. J Mol Evol 63:54–68.

Bustamante CD, Fledel-Alon A, Williamson S, Nielsen R, Hubisz MT, Glanowski S, Tanenbaum DM, White TJ, Sninsky JJ, Hernandez RD, et al. 2005. Natural selection on protein-coding genes in the human genome. Nature 437:1153–1157.

Cann HM, de Toma C, Cazes L, Legrand MF, Morel V, Piouffre L, Bodmer J, Bodmer WF, Bonne-Tamir B, Cambon-Thomsen A, et al. 2002. A human genome diversity cell line panel. Science 296:261–262.

Chambliss KL, Hinson DD, Trettel F, Malaspina P, Novelletto A, Jakobs C, Gibson KM. 1998. Two exon-skipping mutations as the molecular basis of succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria). Am J Hum Genet 63:399–408.

Dervent A, Gibson KM, Pearl PL, Salomons GS, Jakobs C, Yalcinkaya C. 2004. Photosensitive absence epilepsy with myoclonias and heterozygosity for succinic semialdehyde dehydrogenase (SSADH) deficiency. Clin Neurophysiol 115:1417–1422.

Evans PD, Anderson JR, Vallender EJ, Gilbert SL, Malcom CM, Dorus S, Lahn BT. 2004. Adaptive evolution of ASPM, a major determinant of cerebral cortical size in humans. Hum Mol Genet 13:489–494.

Evans PD, Gilbert SL, Mekel-Bobrov N, Vallender EJ, Anderson JR, Vaez-Azizi LM, Tishkoff SA, Hudson RR, Lahn BT. 2005. Microcephalin, a gene regulating brain size, continues to evolve adaptively in humans. Science 309:1717–1720.

Excoffier L, Laval G, Schneider S. 2005. Arlequin version 3-0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics online 1:47–50.

Gibson KM, Hoffmann GF, Hodson AK, Bottiglieri T, Jakobs C. 1998. 4-Hydroxybutyric acid and the clinical phenotype of succinic semialdehyde dehydrogenase deficiency, an inborn error of GABA metabolism. Neuropediatrics 29:14–22.

Hogema BM, Gupta M, Senephansiri H, Burlingame TG, Taylor M, Jakobs C, Schutgens RB, Froestl W, Snead OC, Diaz-Arrastia R, et al. 2001. Pharmacologic rescue of lethal seizures in mice deficient in succinate semialdehyde dehydrogenase. Nat Genet 29:212–216.

Kidd JR, Black FL, Weiss KM, Balazs I, Kidd KK. 1991. Studies of three Amerindian populations using nuclear DNA polymorphisms. Hum Biol 63:775–794.

Klopfstein S, Currat M, Excoffier L. 2006. The fate of mutations surfing on the wave of a range expansion. Mol Biol Evol 23:482–490.

Kohlrausch FB, Callegari-Jacques SM, Tsuneto LT, Petzl-Erler ML, Hill K, Hurtado AM, Salzano FM, Hutz MH. 2005. Geography influences microsatellite polymorphism diversity in Amerindians. Am J Phys Anthropol 126:463–470.

Malaspina P, Roetto A, Trettel F, Jodice C, Blasi P, Frontali M, Carella M, Franco B, Camaschella C, Novelletto A. 1996. Construction of a YAC contig covering human chromosome 6p22. Genomics 36:399–407.

Mekel-Bobrov N, Gilbert SL, Evans PD, Vallender EJ, Anderson JR, Hudson RR, Tishkoff SA, Lahn BT. 2005. Ongoing adaptive evolution of ASPM, a brain size determinant in *Homo sapiens*. Science 309:1720–1722.

Murphy TC, Amarnath V, Gibson KM, Picklo Sr MJ. 2003. Oxidation of 4-hydroxy-2-nonenal by succinic semialdehyde dehydrogenase (ALDH5A). J Neurochem 86:298–305.

Plomin R, Turic DM, Hill L, Turic DE, Stephens M, Williams J, Owen MJ, O'Donovan MC. 2004. A functional polymorphism in the succinate-semialdehyde dehydrogenase (aldehyde dehydrogenase 5 family, member A1) gene is associated with cognitive ability. Mol Psychiatry 9:582–586.

Sokal RR, Oden NL. 1978. Spatial autocorrelation analysis in biology. I. Methodology. Biol J Linn Soc 10:199–228.

Stephens M, Smith NJ, Donnelly P. 2001. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978–989.

Thompson EE, Kuttab-Boulos H, Witonsky D, Yang L, Roe BA, Di Rienzo A. 2004. CYP3A variation and the evolution of salt-sensitivity variants. Am J Hum Genet 75:1059–1069.

Vander Molen J, Frisse LM, Fullerton SM, Qian Y, Del Bosque-Plata L, Hudson RR, Di Rienzo A. 2005. Population genetics of CAPN10 and GPR35: Implications for the evolution of type 2 diabetes variants. Am J Hum Genet 76:548–560.

Voight BF, Kudaravalli S, Wen X, Pritchard JK. 2006. A map of recent positive selection in the human genome. PLoS Biol 4:e72.

Wang YQ, Su B. 2004. Molecular evolution of microcephalin, a gene determining human brain size. Hum Mol Genet 13:1131–1137.

4. Conclusive remarks and perspectives

Succinic semialdehyde dehydrogenase is a crucial enzyme in the brain metabolism which catalyzes the last step of the GABA shunt pathways. In humans, mutations leading to a dramatic SSADH deficiency result in a metabolic disorder that leads to a variable clinical phenotype, ranging from mild retardation in psychomotor and language development to more severe neurological defects. Furthermore, it is has been shown that a low efficiency of the SSADH enzyme induces oxidative stress either by inefficient aldehyde(s) clearance or through increased concentrations of GHB in tissues and organs. In view of the key role of this enzyme in nervous system, and considering the variability of symptoms in SSADH-deficient patients, it has been hypotized that normal variation of *SSADH* gene can be associated with subclinical phenotypes and, perhaps, with cognitive or behavioural variability. In this context the functional polymorphism C538T in the exon 3 of the *SSADH* gene seems to be very interesting for many reasons. The enzyme activity of the Tyr_{180} protein encoded by the 538T allele is 82.5 % of the activity of the His_{180} protein encoded by allele 538C. A study carried out in unrelated subjects and within families, showed that the 538C allele was associated with increased cognitive performance, thus suggesting that higher SSADH activity is associated with higher cognitive performance across the general population. Interestingly, the 538C allele, which is by far the more frequent in the human population, is not present among non human primates, suggesting it has been acquired by the human evolutionary lineage.

On the basis of these observations, we investigated if the *SSADH* C538T polymorphism affects the variability of the cognitive decline in the elderly, due to the effect of SSADH deficiency on cognitive performance and on oxidative stress. On the other hand, given the importance of the phenotypes influenced by *SSADH* C538T polymorphism, we investigated if there is any sign of natural selection favouring one allele, as it has been shown for other genes affecting cognitive phenotypes such as *microcephalin.*

In order to test the hypothesis that the *SSADH* C538T polymorphism plays a role in the variability of the cognitive decline in the elderly, we studied the association between the C538T polymorphism and preservation of cognitive function in the elderly in a sample population from southern Italy. In addition, given the central role of cognitive conservation for survival in the elderly, we studied the effects of this polymorphism on survival in the

same population. We found that, within the 65-85 years age range, the TT genotype is overrepresented in subjects with impaired cognitive function compared to those with conserved cognitive function. Furthermore, we found that the TT genotype affects survival after 65 years of age. In fact, after this age, the survival function of TT homozygous subjects is lower than that of the others. Our results clearly suggest that the efficiency of the SSADH enzyme is important for the preservation of cognitive function and survival in the elderly.

In order to search for possible signatures of natural selection on *SSADH* gene, we assayed worldwide frequencies of the two non-synonymous variants C545T and C538T. We found a clear geographic pattern of analyzed *SSADH* variants, with high frequencies of the ancestral haplotype in Africa, contrasting with high frequencies of derived variant out of Africa. We explored the covariation across human populations with another marker for which strong evidence of positive selection was produced, i.e. MCPH1, and we found a significative correlation between the frequencies of the derived alleles in the two genes. These data are in agreement with the hypothesis of a positive natural selection on allele 538C of *SSADH* gene, such as *microcephalin* and other similar genes.

On the whole our results confirm that the common *SSADH* C538T polymorphism has important effects on the common variability of cognitive-related phenotypes and that this may have produced a positive natural selection on 538C allele within the human species. This may open new perspectives for the study of the effects of common genetic polymorphisms of brain related molecules on cognitive and behavioural variability. In this frame, it will be particularly important to study the effect of these polymorphisms on the cognitive performances of the elderly, considering that the age related accumulation of oxidative damages may exacerbate the slight phenotypic differences due to the genetic variability. Thus, to elucidate the effect of genetic polymorphisms in brain related molecules may help to uderstand the individual variablity of cognitive decline which is one of the most important aspects of age related decline. Given the continuous increase of the elderly population, these researches may also have an enourmous importance both from a social and from a medical point of view.

On the other hand our results confirm the necessity to study these molecules from an evolutionary point of view, as their variability may have played a very important role on the human evolution, and in particular on the evolution of specifically human traits. This is now greatly aided by the availability of high density SNP typing (International Hap Map

Consortium), which enables the investigation of patterns on linkage disequilibrium in populations of different ethnic origin. A relevant subset of the same SNPs has been recently typed in the entire HGDP panel and, when merged with our data, this resource will immediately enable the reconstruction of haplotypes in *cis* to the C538T variants. This would certainly shed a light on the evolution each allele has gone through during human history.

5. References

J. Ahn, T.W. Won, A. Zia, H. Reutter, D.E. Kaplan, R. Sparks, J.R. Gruen (2001). Peaks of linkage are localized by a BAC/PAC contig of the 6p reading disability locus. Genomics, 78: 19-29.

S. Akaboshi, B.M. Hogema, G.S. Salomons, C. Jakobs, P. Malaspina, A. Novelletto, M. Grompe, K.M. Gibson (2001). Identification and in vitro expression of mutant succinic semialdehyde dehydrogenase (SSADH) alleles. J. Inher. Metab. Dis., 24: 63-72.

S. Akaboshi, B.M. Hogema, A. Novelletto, P. Malaspina, G.S. Salomons, G.D. Maropoulos, C. Jakobs, M. Grompe, K.M. Gibson (2003). Mutational spectrum of Succinate Semialdehyde Dehydrogenase (ALDH5A1) gene and functional analysis of 27 novel disease-causing mutation in patients with SSADH deficienc. Hum. Mutat., 22: 442-450.

T.M. Anderson, P.S. Sachdev, H. Brodaty, J.N. Trollor, G. Andrews (2007). Effects of sociodemographic and health variables on mini-mental state exam scores in older Australians. Am. J. Geriatr. Psychiatry, 15: 467-476.

M. Bamshad, S.P. Wooding (2003). Signatures of natural selection in the human genome. Nat. Rev. Genet., 4: 99-111.

L. Bathum, L. Christiansen, B. Jeune, J. Vaupel, M. McGue, K. Christensen (2006). Apolipoprotein E genotypes: relationship to cognitive functioning, cognitive decline, and survival in nonagenarians. J. Am. Geriatr. Soc., 54: 654-658.

L. Bathum, L., J. von Bornemann Hjelmborg, L. Christiansen, M. McGue, B. Jeune, K. Christensen (2007). Methylenetetrahydrofolate reductase 677C>T and methionine synthase 2756A>G mutations: no impact on survival, cognitive functioning, or cognitive decline in nonagenarians. J. Gerontol. A. Biol. Sci. Med. Sci., 62 : 196-201.

P. Blasi, P. Pilo Boyl, M. Ledda, A. Novelletto, K.M. Gibson, C. Jakobs, B. Hogema, S. Akaboshi, F. Loreni, P. Malaspina (2002). Structure of human succinic semialdehyde dehydrogenase gene: identification of promoter region and alternatively processed isoforms. Molec. Genet. Metab., 76: 348-362.

P. Blasi, F. Palmerio, A. Aiello, M. Rocchi, P. Malaspina (2006). SSADH variations in Primates: intra- and interspecific data on a gene with a potential role in human cognitive functions. J. Mol. Evol., 63: 54-68.

S. Bekri, C. Fossoud, G. Plaza, A. Guenne, G.S. Salomons, C. Jacobs, E. Van Obberghen (2004). The molecular basis of succinic semialdehyde dehydrogenase deficiency in one family. Mol. Genet. and Metab., 81: 347-351.

G.K. Brown, C.H. Cromby, N.J. Manning, R.J. Polliti (1987). Urinary organic acids in succinic semialdehyde dehydrogenase deficiency: evidence of alpha-oxidation of 4-hydroxybutiric acid, interaction of succinic semialdehyde with pyruvate dehydrogenase and possible secondary inhibition of mitochondrial beta-oxidation. J. Inherit. Metab. Dis., 10: 367-375.

C.D. Cash, M. Maitre, P. Mandel. (1979) Purification from human brain and some properties of two NADPH-linked aldehyde reductases with reduce succinic semialdehyde to 4-hydroxybutyrate. J. Neurochem., 33: 1169-1175.

C.D. Cash (1994). Gammahydroxybutyrate: an overview of the pros and cons for it being a neurotransmiteter and/or a useful therapeutic agent. Neurosci. Biobehav. Rev., 18: 291-304.

K.L. Chambliss and K.M. Gibson (1992). Succinic semialdehyde dehydrogenase from mammalian brain: subunit analysis using polyclonal antiserum. Int. J. Biochem., 24: 1493-1499.

K.L. Chambliss, D.L. Caudle, D.H. Hinson, C.R. Moomaw, C.A. Slaughter, C. Jakobs, M.K. Gibson (1995). Molecular cloning of the mature NAD+-dependent succinic semialdehyde dehydrogenase from rat and human. J. Biol. Chem., 270: 461-467.

K.L. Chambliss, D.D. Hinson, F. Trettel, P. Malaspina, A. Novelletto, C. Jacobs, K.M. Gibson (1998). Two exon-skipping mutations as the molecular basis of succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria). Am. J. Hum. Genet., 63: 399-408.

N. Cope, D. Harold, G. Hill, V. Moskvina, J. Stevenson, P. Holmans, M.J. Owen, M.C. O'Donovan, J. Williams (2005). Strong evidence that KIAA0319 on chromosome 6p is a susceptibility gene for developmental dyslexia. Am. J. Hum. Genet., 76: 581-591.

M.A. Cortez, W.Y. Gibson, O.C. Snead (2004). Absence seizures in succinic semialdheyde dehydrogenase deficient mice: a model of juvenile absence epilepsy. Pharmacol. Biochem. Behav. 79: 547-553.

S. Dato, L. Carotenuto, G. De Benedictis (2007). Genes and longevity: a genetic-demographic approach reveals sex- and age-specific gene effects not shown by the case-control approach (APOE and HSP70.1 loci). Biogerontology, 8: 31-41.

K.E. Deffenbacher, J.B. Kenyon, D.M. Hoover, R.K. Olson, B.F. Pennington, J.C. DeFries, S.D. Smith (2004). Refinement of the quantitative trait locus influencing dislexia: linkage and association analyses. Hum. Genet., 115: 128-38.

C. Dennis (2005). Chimp genome: branching out. Nature, 437: 17-19.

D. De Ronchi, D. Berardi, M. Menchetti, G. Ferrari, A. Serretti, E. Dalmonte, L. Fratiglioni (2005). Occurrence of cognitive impairment and dementia after the age of 60: a population-based study from Northern Italy. Dement. Geriatr. Cogn. Disord., 19: 97-105.

A. Dervent, K.M. Gibson, P.L. Pearl, G.S. Salomons, C. Jacobs, C. Yalcinkaya (2004). Photosensitive absence epilepsy with myoclonias and heterozygosity for succinic semialdehyde dehydrogenase (SSADH) deficiency. Clin. Neurophysiol., 115: 1417-1422.

D.C. De Vivo, K.M. Gibson, L.D. Resor, M. Steinschneider, S. Aramaki, L. Cote (1988). 4-hydroxybutyric aciduria: clinical features, pathogenetic mechanism, and treatment strategies. Ann. Neurol., 24: 304 – 309.

S. Dorus, E.J. Vallender, P.D. Evans, J.R. Anderson, S.L. Gilbert, M. Mahowald, G.J. Wyckoff, C.M. Malcom, B.T. Lahn (2004). Accelerated evolution of nervous system genes in the origin of *Homo sapiens*. Cell, 119: 1027-1040.

W. Enard, M. Przeworski, S.E. Fisher, C.S. Lai, V. Wiebe, T. Kitano, A.P. Monaco, S. Paabo (2002). Molecular evolution of FOXP2, a gene involved in speech lamguage. Nature, 418: 869-872.

P.D. Evans, J.R. Anderson, E.J. Vallender, S.S. Choi, B.T. Lahn (2004). Reconstructing the evolutionary history of *microcephalin*, a gene controlling human brain size. Hum. Mol. Genet., 13: 1139-1145.

P.D. Evans, S.L. Gilbert, N. Mekel-Bobrov, E.J. Vallender, J.R. Anderson, L.M. Vaez-Azizi, S.A. Tishkoff, R.R. Hudson, B.T. Lahn (2005). *Microcephalin*, a gene regulating brain size, continues to evolve adaptively in humans. Science, 309: 1717-1720.

M.F. Folstein, S.E. Folstein, P.R. McHugh (1975). "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res., 12: 189-198.

K.M. Gibson, L. Sweetman, W.L. Nyhan, C. Jakobs, D. rating, H. Siemes, F. Hanefeld. (1983). Succinic semialdehyde dehydrogenase deficiency: an inborn error of gamma-aminobutyric acid metabolism. Clin. Chim. Acta, 133: 33-42.

K.M. Gibson, C.F. Lee, K.L. Chambliss, V. Kamali, B. Francois, J. Jaeken, C. Jakobs (1991). 4-Hydroxibutyric aciduria: application of a fluorometric assay to the determination of succinic semialdehyde dehydrogenase activity in extracts of cultured human lymphoblasts. Clin. Chim. Acta, 196: 219-221.

K.M. Gibson, C. Baumann, H. Ogier, E. Rossier, B. Vollmer, C. Jacobs (1994). Pre- and postnatal diagnosis of succinic semialdehyde dehydrogenase deficiency using enzyme and metabolite assays. J. Inherit. Metab. Dis., 17: 732-737.

K.M. Gibson, E. Christensen, C. Jakobs, B. Fowler, M.A. Clarke, G. Hammersen, K. Raab, et al. (1997). Clinical phenotype of succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria): case reports of 23 new patients. Pediatrics, 99: 567-574.

K.M. Gibson, C. Jakobs (2001). Disorders of beta- and gamma-aminoacids in free and peptide-linked forms. In: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. valle (Eds), the Metabolic and molecular Bases of Inherited Disease, eighth ed., McGraw-Hill, New York, pp. 2079-2105.

K.M. Gibson, D.S.M. Schor, M. Gupta, W.S. Guerand, H. Senephansiri, T.G. Burlingame, H. Bartels, B.M. Hogema, T. Bottiglieri, W. Froestl, O.C. Snead, M. Grompe, C. Jacobs (2002). Focal neurotransmitter alterations in mice deficient for succinate semialdehyde dehydrogenase. J. Neurochem., 81: 71-79.

K.M. Gibson, M. Gupta, P.L. Pearl, M. Tuchman, L.G. Vezina, O. Snead, L.M. Smith, C. Jacobs (2003). Significant behavioural disturbances in succinico semialdehyde dehydrogenase (SSADH) deficiency (gamma-hydroxybutyric aciduria). Biol. Psychiatry, 54: 763-768.

K.M. Gibson (2005). Gamma-hydroxybutyric aciduria: a biochemist's education from a heritable disorder of GABA metabolism. J. Inherit. Metab. Dis., 28: 247-265.

P. Gilon and C. Remacle (1992). GABA and endocrine cells of the gastro-entero-pancreatic system: a review. In GABA outside the CNS (edited by Erdo S.L.), 111-132. Springer-Verlag, New York.

F. Grigoletto, G. Zappalà, D.W. Anderson, B.D. Lebowitz (1999). Norms for the Mini-Mental State Examination in a healthy population. Neurology, 53: 315-320.

M. Gupta, M. Polinsky, H. Senephansiri, O.C. Snead, E.E.W. Jansen, C. Jacobs, K.M. Gibson (2004). Seizure evolution and amino acid imbalance in murine succinate semialdehyde dehydrogenase (SSADH) deficiency. Neurobiol. Dis., 16: 556-562.

B.M. Hogema, M. Gupta, H. Senephansiri, T.G. Burlingame, M. Taylor, C. Jakobs, R.B. Schutgens, W. Froestl, O.C. Snead, R. Diaz-Arrastia, T. Bottiglieri, M. Grompe, K.M. Gibson (2001). Pharmacological rescue of lethal seizure in mice deficient in succinate semialdehyde dehydrogenase. Nat. Genet., 29: 212-216.

C. Jacobs, M. Bojasch, E. Monch, D. Rating, H. Siemens, F. Hanefeld (1981). Urinary excretion of gamma-hydroxybutyric acid in a patient with neurological abnormalities. The probability of a new inborn error of metabolism. Clin. Chim. Acta, 11: 169-178.

C. Jacobs, L.M. Smit, J. Kneer, T. Michael, K.M. Gibson (1990). The first adult case with 4-hydroxybutyric aciduria. J. Inherit. Metab. Dis., 13: 341-344.

J.N. Keller, R.J. Mark, A.J. Bruce, E Blanc, J.D Rothstein, J. Uccida, G. Waeg, M.P. Mattson. (1997). 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. Neuroscience, 80: 685-696.

D.I.B. Kerr and J. Ong (1992). GABA and gut motility. In GABA outside the CNS (Edited by Erdo S.L.), 29-44. Springer-Verlag, New York.

E.R. Londin, H. Meng and J.R. Gruen (2003). A transcription map of the 6p22.3 reading disability locus identifying candidate genes. Genomics, 4: 25- 32.

V.D. Longo and P. Fabrizio, 2002. Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans? Cell. Mol. Life Sci., 59: 903–908.

S. Lorenz, A. Heils, K.P. Taylor, A. Gehrmann, H. Muhle, M. Gresch, T. Becker, U. Tauer, U. Stephani, T. Sander. (2006). Candidate gene analysis of the succinic semialdehyde dehydrogenase gene (ALDH5A1) in patients with idiopathic generalized epilepsy and photosensitivity. Neurosc. Letters, 397: 234-239.

E. Magni, G. Binetti, A. Bianchetti, R. Rozzini, M. Trabucchi (1996). Mini-mental state examination: a normative study in Italian elderly population. Eur J Neurol., 3: 1-5.

M. Maitre (1997). The gamma-hydroxybutirate signalling system in brain: organization and functional implications. Progr. Neurobiol., 51: 337-361.

E. McKracken, D.I. Graham, M. Nilsen, J. Stewart, J.A. Nicoll, K. Horburg (2001). 4-Hydroxynonenal immunoreactivity is increased in human hippocampus after global ischemia. Brain Pathol., 11: 414-421.

P. Malaspina, A. Roetto, F. Trettel, C. Jodice, P. Blasi, M. Frontali, M. Carella, B. Franco, C. Camaschella, A. Novelletto (1996). Construction of a YAC contig covering human chromosome 6p22. Genomics, 36: 399-407.

A.K. Mehta, M. Gupta, K.M. Gibson (2002). Decreased GHB and GABA receptor building in succinate semialdehyde dehydrogenase deficient mouse brain. Program No. 431.415. Abstract Viewer/Itinerary Planner. Online. Washington, DC: Society for Neuroscience. www.sfn.org.

M.J. Meyer, D.E. Mosely, V. Amarnath, M.J. Picklo (2004). Metabolism of 4-Hydroxy-*trans*-2-nonenal by central nervous system mitochondria is dependent on age and NAD+ availability. Chem. Res. Toxicol., 17: 1272-1279.

T.C. Murphy, V. Amarnath, J.M. Gibson, M.J. Picklo (2003). Oxidation of 4-hydroxy-2-nonenal by succinic semialdeide dehydrogenase (ALDH5A). J. Neurochem., 86: 298-305.

P. Neu, S. Seyfert, J. Brockmoller, M. Dettling, P. Marx (2002). Neuroleptic malignant syndrome in a patient with semialdehyde dehydrogenase deficiency. Pharmacopsychiatry, 35: 26-28.

J.G. Okun, S. Sauer, G.F. Hoffmann, T.G. Burlingame, S. Koelker, K.M. Gibson (2003). Inhibition of ubiquinone-dependent respiratory chain complexes by 4,5-dihydroxyhexanoic acid in succinate semialdehyde dehydrogenase deficiency. J. Inherit. Metab. Dis. 26: 53- 61.

T. Perls, L.M. Kunkel, A.A. Puca (2002). The genetics of exceptional human longevity. J. Am. Geriatr. Soc., 50: 359-368.

P.L. Pearl, K.M. Gibson, M.T. Acosta, L.G. Vezina, W.H. Theodore, M.A. Rogawski, E.J. Novotny, A. Gropman, J.A. Conry, G.T. Berry, M. Tuchman (2003a). Clinical spectrum of succinic semialdehyde dehydrogenase deficiency. Neurology, 60: 1423-1417.

P.L. Pearl, E.J. Novotny, M.T. Acosta, C. Jacobs, K.M. Gibson (2003b). Succinic Semialdehyde Dehydrogenase Deficiency in children and adults. Ann. Neurol., 54: S73-S80.

P.L. Pearl, P.K. Capp, E.J. Novotny, K.M. Gibson (2005). Inherited disorders of neurotransmitters in children and adults. Clinical Biochem., 38: 1051-1058.

M.J. Picklo, S.J. Olson, W.R. Markesbery, T.J. Montine (2001a). Expression and activities of aldo-keto oxireductases in Alzheimer disease. J. Neuropathol. Exp. Neurol., 60: 686-695.

M.J. Picklo, S.J. Olson, J.D. Hayes, W.R. Markesbery, T.J. Montine (2001b). Elvation of AKR7A2 (succinic semialdhehyde reductase) in neurodegenerative disease. Brain Res., 916: 229-238.

M.J. Picklo, T.J. Montine, V. Amarnath, M.D. Neely (2002). Carbonyl toxicology and Alzheimer's disease. Toxicol. Appl. Pharmacol., 184: 187-197.

R. Plomin, D.M. Turic, L. Hill, D.E. Turic, M. Stephens, J. Williams, M.J. Owen, M.C. O'Donovan (2004). A functional polymorphism in the succinate-semialdehyde dehydrogenase (aldehyde dehydrogenase 5 family, member A1) gene is associated with cognitive ability. Mol. Psychiatry, 9: 582-586.

G. Rait, A. Fletcher, L. Smeeth, C. Brayne, S. Stirling, M. Nunes, E. Breeze, E.S. Ng, C.J. Bulpitt, D. Jones, A.J. Tulloch (2005). Prevalence of cognitive impairment: results from the MRC trial of assessment and management of older people in the community. Age Ageing, 34: 242-248.

M.T. Ryzlak, R. Pietruszko (1988). Human brain „high Km“ aldehyde dehydrogenase: purification, characterization, and identification as NAD+-dependent succinic semialdehyde dehydrogenase. Arch. Biochem. Biophys., 266: 386-396.

S.W. Sauer, S. Kolker, G.F. Hoffmann, H.J. ten Brink, C. Jakobs, K.M. Gibson, J.G. Okun (2006). Enzymatic and metabolic evidence for a region specific mitochondrial dysfunction in brains of murine succinic semialdehyde dehydrogenase deficiency (Aldh5a1$^{-/-}$ mice). Neurochem. Intern., 50: 653-659.

P.C. Sabeti, S.F. Schaffner, B. Fry, J. Lohmueller, P. Varilly, O. Shamovsky, A. Palma, T.S. Tikkelsen, D. Altshuler, E.S. Lander (2006). Positive natural selection in the human lineage. Science, 312: 1614-1620.

A.M. Sgaravatti, M.B. Sgarbi, C.G. Testa, K. Durigon, C.D. Pederzolli, C.C. Prestes, A.T.S. Wyse, C.M.D. Wannmacher, M. Wajner, C.S. Dutra-Filho (2007). γ-hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. Neurochem. Intern., 50: 564-570.

U. Schoerken, G.A. Sprenger (1998). Thiamin-dependent enzymes as catalysts in chemoenzymatic syntheses. Biochim Biophys Acta, 1385: 229-243.

O.C. Snead (1978). Gamma hydroxybutyrate in the monkey. I. Electroencephalographic, behavioral, and pharmacokinetic studies. Neurology, 28: 636-42.

O.C. Snead (2000). Evidence for a G protein-coupled gamma-hydroxyhexanoic acid receptor. J. Neurochem., 75: 1986-1996.

N.A. Sophos, V. Vasiliou (2003). Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem. Biol. Interact., 143-144: 5-22.

N.J.K. Tillakaratne, L. Medina-Kauwe and K.M. Gibson. (1995). Gamma-aminobutyric acid (GABA) metabolism in mammalian neural and nonneural tissues. Comp. Biochem.Physiol., 122: 247-263.

G. Thomas, S. Bonner, A. Gascoigne (1997). Coma induced by abuse of gamma-hydroxybutyrate (GHB or liquid ecstasy): a case report. BMJ, 314: 35-36.

V. Vasiliou, A. Bairoch, J.K. Tipton, D.W. Nebert (1999). Eukaryotic aldehyde dehydrogenase (ALDH) genes: human polymorphisms, and recommended nomenclature based on divergent evoluition and chromosomal mapping. Pharmacogenetics, 9: 421-434.

H.S. Waagepetersen, U. Sonnewald and A. Schousboe. (1999). The GABA Paradox: Multiple Roles as Metabolite, Neurotransmitter, and Neurodifferentiative Agent. J. Neurochem., 73: 1335-1342.

J.R. Wolff, F. Joo, W. Dames (1978). Plasticity in dendrites shown by continuous GABA administration in superior cervical ganglion of adult rat. Nature, 274: 72-74.

A. Yoshida, A. Rzhetsky, L.C. Hsu, C. Chang (1998). Human aldehyde dehydrogenase gene family. Eur. J. Biochem., 251: 549-557.

B. Zhang, Y. Yanbo, J. Yanbin, Y. Xin, X. Qi, S. Yucun, S. Yan. (2005) An association study between polymorphisms in five genes in glutamate and GABA patway and paranoid schizophrenia. Europ. Psychiatry, 20: 45-49.