PATHOPHYSIOLOGICAL PROCESSES AND POSSIBLE THERAPEUTICAL STRATEGIES IN NEURODEGENERATION. 
CLINICAL AND BASIC STUDIES

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1. INTRODUCTION:

1.1. STUDY BACKGROUND

Socio-economic impact of neurodegenerative diseases

Most neurodegenerative diseases are age-related, thus representing a relevant socio-economic problem in particular for industrialized countries. Alzheimer's (AD) and Parkinson's Disease (PD) represent two of the most frequent clinical expressions of neurodegeneration.

Genetic and environmental factors in neurodegenerative disorders

Great genetic heterogeneity represents an important aspect of neurodegeneration: some diseases are multifactorial, both hereditary and sporadic, whereas others are monogenic. Therefore, we can observe cases with Mendelian transmission and complete penetrance, and others in which genetics predispose to greater disease susceptibility. In this scenery, the influence of environmental factors (such as heavy metals, pesticides, nutrition, head trauma, and infections) plays a fundamental role in contributing to disease development.

The increasing focus on genetic etiology of diseases over the past 20 years has resulted in a significant body of knowledge for neurological pathologies. Specifically, many movement disorders previously considered “idiopathic” have been shown to have a significant genetic contribution to etiology, ranging from single gene dysfunction to complex interaction between risk genes and environment. Noteworthy, the advances in the genetics of parkinsonism have provided great insight not only into the role of genetics in sporadic PD, but also into the molecular pathways and disease mechanisms involved in the pathogenesis of the so-called “idiopathic” syndrome.

Alzheimer's Disease wise, positional cloning led to the identification of rare, disease-causing mutations in APP, PSEN1, and PSEN2 responsible for early-onset familial AD, followed by the discovery of APOE as the single most important risk factor for late-onset AD. It is however interesting to notice how during the course of only three years, genome-wide association studies (GWAS) in AD have yielded more reproducible, consistent and thus likely more relevant findings than three decades of candidate-gene-driven research. Recent GWAS have delivered several additional AD susceptibility loci that are common in the general population, but exert only very small risk effects. As a result, a large proportion of the heritability of AD continues to remain unexplained by the currently known disease genes.

Hopefully, future genetic research and the identification of pathways involved in neurodegenerative disease, might pave the way for early diagnosis and new therapeutic targets at these pathways.
1.2. THESIS SUMMARY

From the above considerations, our research interest began from a clinical phase, proceeding through two ensuing experimental projects.

They did proceed independently, pointing to genetics in AD and genomics in PD respectively: if in the first project a specific interesting gene has emerged as possibly related to AD, and further validation analysis is already going on, in the second study at date we have preliminary gene expression profiling findings, pointing to both “central” and “peripheral” pathways involved in PD neurodegeneration.

Project 1: Genome-wise association study - Genetic Park Project Friuli Venezia Giulia

The first study, whose core is the whole genome analysis, involves all inhabitants of six isolated populations of Friuli Venezia-Giulia.

Potentially, it offers a powerful opportunity for evaluating and understanding the relative contribution of genes and environment in the development of complex or multifactorial diseases, including neurodegenerative disorders and among them Alzheimer’s Disease in particular.

The identification of susceptibility genes for this subset of pathologies is very challenging, but research performed in isolated populations might offer unique information, thanks to favourable characteristics such as reduced genetic heterogeneity secondary to “founder effect” and endogamia, and greater environmental uniformity, as compared with large continental populations.

This study can be subdivided into three phases:

1. data collection of possible risk factors (nutrition, habits, head trauma, toxic exposition, and also cardiovascular risk factors), family recurrence of cognitive impairment, personal history of cognitive impairment
2. clinical screening, through simple but specific cognitive and neurological evaluations, aiming at identification of possible AD cases
3. blood collection and genome analysis in the group of subjects with clinical suspicion of AD with/without cerebrovascular disease.

Epidemiological data analysis evidenced greater dementia prevalence in isolated populations if compared to prevalence in Italian population (9% versus 5%, in people >65 years).

After whole genome analysis and comparisons of genetic data of healthy and affected subjects, possible correlations between dementia and some specific risk factors (low education, atrial fibrillation and hypertension) have been found.

Finally, Genome Wise Association Study finally found a really interesting gene differently expressed in demented patients: PTPRD gene on chromosome 9, that is already know to play an important role in Long Term Potentiation mechanisms in mice. To our knowledge, this is the first
report of possible PTPRD involvement in humans with cognitive decline, and the first from GWAS to be somehow not related to Aβ hypothesis.

*Project 2: Gene expression profiling in de novo Parkinson’s Disease patients*

The second study intends to analyze gene expression profiling in patients with a clinical diagnosis of PD, confirmed by DaTSCAN, naïve from any specific therapy. We performed a transcriptome-wide scan in 40 idiopathic naïve PD patients, looking for molecular processes alteration in their blood, cells, and comparing the results with those of 20 healthy controls, matched for age, sex and environmental factors.

At date, two “black holes” in Parkinson’s Disease management are particularly important for clinicians, and are consequently among research challenges: firstly, there’s no available laboratory test correlating with PD risk, therefore useful for early diagnosis; secondly, the absence of disease-modifying or neuroprotective treatments. The aim of this project is to find out potential biomarkers for PD, easily detectable in peripheral blood through non-invasive methods, and possibly allowing a pre-clinical diagnosis.

Instead of testing all possible mutated genes in PD, microarrays used in this experimental protocol allow to obtain a systematic scan of genome-wide expression changes in blood cells, thus evaluating in parallel expression levels of thousands of genes.

After clinical diagnosis and instrumental confirmation of PD, the experimental design includes RNA isolation from venous whole blood, microarray analysis, data processing, Real-Time PCR, functional classification process.

According to preliminary results of gene expression profiling in PD patients, some specific pathways seem to be related to PD pathological processes, in particular: hypoxia, axonal guidance, calcium signaling, inflammation, diabetes, glycosphingolipid metabolism, neurotransmitters pathways, neurodegenerative diseases.

Further studies and replication of these findings are obviously mandatory for their validation, but we think this could be a starting point for understanding physiopathological mechanisms and developing new therapeutical strategies in Parkinson’s Disease.
2. NEURODEGENERATION

2.1. DEFINITION:

Neurodegeneration is a commonly used word whose meaning is supposed to be universally understood; yet finding a precise definition for neurodegeneration is not easy. Etymologically, the word is composed of the prefix “neuro-,” which designates nerve cells (neurons), and “degeneration,” which refers to a process of losing structure or function.

Thus, in the strict sense of the word, neurodegeneration corresponds to any pathological condition primarily affecting neurons. In practice, neurodegenerative diseases represent a large group of neurological disorders with heterogeneous clinical and pathological expressions, affecting specific subsets of neurons in specific functional anatomic systems; they arise for unknown reasons and progress in a relentless manner (Przedborski et al, 2003).

There are many parallels between different neurodegenerative disorders (i.e. atypical protein assemblies, induced cell death), but still a wide spectrum of mechanisms and pathways underlying neurodegeneration: inflammation (Glass et al, 2010), immune response (Lucin et al, 2009), mitochondrial function (Morais et al, 2010), metalloproteinase (Rosenberg et al, 2009), axonal transport (Morfini et al, 2009) and prion-like mechanisms (Brundin et al, 2010) are thought to be the most relevant ones.

Many neurodegenerative diseases are caused by genetic mutations, most of which are located in completely unrelated genes. For example, in diseases known as polyglutamine diseases like Huntington's Disease (HD), the mutated gene has a common feature: a repeat of the CAG nucleotide triplet, resulting in a polyglutamine (polyQ) tract. Extra glutamine residues can acquire toxic properties through irregular protein folding and degradation pathways, altered subcellular localization, and abnormal interactions with other cellular proteins.

While polyQ diseases encompass many different neurodegenerative disorders, there are many more it does not apply to. The genetics behind each disease are different and often unknown, mostly because they represent only a predisposition background, susceptible to environmental influence.

Parkinson's disease and Huntington's disease are both late-onset and associated with the accumulation of intracellular toxic proteins. Diseases caused by the aggregation of proteins are known as proteinopathies, and they are primarily caused by aggregates in different structures including cytosol (e.g. PD and HD), nucleus (e.g. Spinocerebellar ataxia type 1, SCA1), endoplasmic reticulum (ER), extracellularly excreted proteins (e.g. Aβ in AD) (Rubinsztein et al, Nature 2006).

There are two main ways used by cells to remove these troublesome proteins, the ubiquitin–proteasome system and the autophagy-lysosome pathway (Martin et al, 2010).
In the first case, ubiquitin along with enzymes is key for the degradation of many proteins that cause proteinopathies, including polyQ expansions and alpha-synucleins. The second pathway is a form of programmed cell death (PCD), which becomes the favorable route when a protein is a poor proteasome substrate. Programmed cell death (PCD) is death of a cell in any form, mediated by an intracellular program. There are, however, situations in which these mediated pathways are artificially stimulated due to injury or disease (Bredesen et al, 2006).

There are two forms of autophagy:

- Macroautophagy is involved with nutrient recycling of macromolecules under conditions of starvation, certain apoptotic pathways, and if absent, leads to the formation of ubiquinated inclusions.
- Chaperone-mediated autophagy (CMA) defects may also lead to neurodegeneration when mutant proteins bind to the CMA-pathway receptors on lysosomal membrane, thus blocking their own degradation as well as the degradation of other substrates.

Neuronal death in neurodegenerative disorders could be the consequence of apoptosis or necrosis. In both processes—but mainly in apoptosis—the caspase (cysteine-aspartic acid proteases) family of proteases is likely to have an important role (Avila et al, 2010). Caspases have been classified into initiator, like caspase 9, and executioner proteins, like caspases 3 and 6.

In most common form of apoptotic cell death in neurodegeneration, that is the intrinsic mitochondrial pathway, the release of cytochrome c from the mitochondrial intermembrane space controls the activation of caspase 9 (Avila et al, 2010; de Calignon et al, 2010). Executioner caspases could be activated upstream by the presence of extracellular factors, such as Aβ peptide (Gamblin et al, 2003) and could act downstream on several substrates, one of which is the tau protein. Caspase cuts the tau molecule at position 421 (Asp421) in the absence of Ser422 phosphorylation, yielding a truncated tau molecule (Berry et al, 2003) that facilitates tau aggregation and neurofibrillary tangles (NFTs) formation. Presence of NFTs correlates with a decrease in caspase activation that leads to cell survival.

A possible hypothesis on the relationship between caspases, NFTs and cell death is that in neurons that bear tangles, cell survival will be facilitated by a decrease in caspase activity. However, NFT accumulation in the neuron may leave little space for organelle function, ultimately leading to cell death, after which the intracellular tangles will become extracellular NFTs (eNFTs). By contrast, if truncation of tau is prevented by phosphorylation, the executioner caspase will remain active, possibly facilitating neurodegeneration (Avila et al, 2010).

Another aspect of mitochondrial pathway involvement in apoptosis is through reactive oxygen species (ROS), normal products of mitochondrial respiratory chain activity, whose over production (oxidative stress) is a central feature of all neurodegenerative disorders. In addition to the generation of ROS, mitochondria are also involved in life-sustaining functions including calcium homeostasis and programmed cell death (PCD). Mitochondrial disease leading to
neurodegeneration is likely, at least on some level, to involve all of these functions (Di Mauro et al, 2008).

There is strong evidence that also axonal transport plays a causal role in neurodegenerative diseases as axonal swelling and spheroids have been observed in many of them. When axonal transport is severely disrupted, a degenerative pathway known as Wallerian degeneration is often triggered (Coleman et al, 2010).

Apoptosis is only one of the main types of programmed cell death (PCD). There are both extrinsic and intrinsic apoptotic pathways: the former occurs when factors outside the cell activate cell surface death receptors which results in the activation of caspases 8 or 10, the latter results from mitochondrial release of cytochrome c or endoplasmic reticulum malfunctions, both of which lead to the activation of caspase-9. The nucleus and Golgi apparatus are other organelles that have damage sensors which can lead the cells down apoptotic pathways.

Other forms of PCD are the previously described autophagy, and the less understood non-apoptotic process called cytoplasmic cell death.

Many other forms of PCD like trophotoxicity, cytotoxins or aponecrosis, are observed but not fully understood or accepted by the scientific community (Bredesen et al, 2006).

Current research, often in transgenic animal models, implicates both apoptotic and non-apoptotic pathways in neurodegeneration, but generally the apoptotic intrinsic mitochondrial pathway is the most frequent (Di Mauro et al, 2008).

2.1. NEURODEGENERATION IN AD

It is useful to view neurodegeneration in AD in the context of neural systems and stages (Braak et al, 1991).

Surprisingly, the classification and mechanisms of neurodegeneration in AD are still not clear. Dying neurons, evidenced with in situ DNA fragmentation/damage assay called TUNEL, are found in subsets of neocortical, hippocampal, and thalamic areas in the AD brain.

Among partially known mechanisms that cause neurodegeneration in AD, there are abnormal processing/modification of APP and the cytoskeletal protein tau, that lead to amyloid (Aβ) deposits and neurofibrillary changes, in the form of paired helical filaments (NFTs) and dystrophic neurites. Upstream to all this, a combination of several mechanisms including perturbations in protein metabolism, excitotoxicity, oxidative stress, mitochondrial dysfunction, and inflammation is possible. Some specific mechanisms for neuronal degeneration in AD may involve dysfunction of NMDA receptors (Sze et al, 2001; Kemp et al, 2001), dysregulation of Ca^{2+} and mitochondrial homeostasis (Reddy et al, 2008), defects in synapses (Selkoe et al, 2002), abnormalities in APP
and presenilines metabolism, toxic actions of Aβ protein (Younkin et al., 1995), and cytoskeletal pathology (Fath et al., 2002).

There are possible disease links between intraneuronal Aβ and mitochondria suggesting an intracellular toxicity of Aβ mediated by interaction with mitochondrial proteins, and consequent bioenergetic deficits (Devi et al., 2006; Manczak et al., 2006).

2.2. NEURODEGENERATION IN PD

PD is a chronically progressive, age-related, neurological disease in humans described first by James Parkinson in 1817.

A major neuropathological feature of PD is the degeneration of dopamine neurons in substantia nigra pars compacta (SNc) and in other brainstem regions. Movement disorder in PD is thought to arise from reduced striatal dopaminergic innervation resulting from the loss of SNc neurons: the effect is over-activity of striatal neurons that project to and inhibit neurons in external globus pallidus (GPe), thus reducing normal GPe inhibition of excitatory subthalamic neurons.

In addition, due to dopamine actions on different dopamine receptor subtypes, there is also loss of dopaminergic excitation of striatal neurons projecting to internal GP (GPI) and SN reticularis, causing increased γ-aminobutyric acidergic inhibition of thalamic nuclei that are needed to drive cortical activation. PD can thus be explained functionally by over-activity of the subthalamic nucleus and GPI.

PD should however be regarded as a multi-system neurodegenerative disorder in which pathology seems to appear in a regionally specific sequence, beginning in the dorsal motor nucleus of the vagus and olfactory bulbs and anterior nucleus followed by the locus coeruleus and then the SNc, at which time (when ~ 50% of SNc neurons are lost) a clinical diagnosis of PD becomes possible (Braak et al., 2003).

The degeneration of pigmented SNc neurons is characterized by chromatolysis (Figure 1A), nuclear condensation (Figure 1B), and severe soma attrition (Figure 1C).

This degeneration pattern could be indicative of autophagy. The nuclear condensation stage of pigmented SNc neuron degeneration is characterized by the appearance of DNA double-strand breaks (Figure 1D), and in the chromatolytic stage SNc neurons accumulate cleaved caspase-3 immunoreactivity (Figure 1E). Another neuropathological feature of PD is the formation of eosinophilic proteinaceous intra-neuronal or intra-glial inclusions (Figure 1F, arrow), known as Lewy Bodies (LBs), usually positive for ubiquitin and α-synuclein (α-Syn) (Goedert et al., 2001).
The molecular pathogenesis of PD is still not understood.

The majority of PD cases are idiopathic (sporadic), while 5-10% is heritable (familial). Epidemiological studies reveal several risk factors for developing idiopathic PD, in addition to aging: pesticides have been linked convincingly to the development of the disease, possibly herbicides, well water (contaminated with pesticides), and industrial chemicals (Schapira et al, 2006).

Considering Mendelian forms of PD, several genes have been so far identified, with autosomal dominant or autosomal recessive inheritance patterns (Table 3). PD-linked mutations occur in the genes encoding α-Syn, Parkin, ubiquitin carboxy-terminal hydrolyase-L1 (UCH-L1), phosphatase and tensin homolog (PTEN)-induced putative kinase-1 (PINK1), DJ-1, and leucine-rich repeat kinase-2 (LRRK2). Genetic aspects of PD will be described later in the text.

2.3. ANIMAL MODELS

2.3.1. ANIMAL MODELS OF ALZHEIMER’S DISEASE:

Animal experiments should provide critical insight into to the mechanisms of neurodegeneration in AD.

However, most transgenic (Tg) mouse models of AD are not useful to study neuronal cell death, as they show modest evidence for neurodegeneration resulting in neuronal cell death, despite massive brain burden of Aβ (Duyckaerts et al, 2008). For example, most human APP
and/or presenilin Tg mice show substantial Aβ deposits in hippocampus and cortex, but do not develop important neuronal loss; none of these studies have found NFTs formation (Takeuchi et al, 2000).

Triple-tg mice with mutant presenilin, APP, and tau have intraneuronal deposits of Aβ and phosphorylated tau, but neuronal loss is not reported (Oddo et al, 2003) Cell culture models of cortical and hippocampal neuron cell death are used to study interactions between APP, Aβ, Tau, and Caspases.

For a long time, it has been thought that Aβ is the primary cause of AD: extracellular application of Aβ can induce apoptosis (Loo et al, 1993) or necrosis (Behl et al, 1994) in cultured neurons.

Studies of the specific intracellular signaling pathways activated by Aβ to trigger cell death are only now appearing in the literature, involving Fas, caspases, p53, and other factors.

It is noteworthy that results of cell culture experiments using extracellular application of Aβ are likely to be dependent on Aβ concentration and oligomeric state. When human neuron primary cultures are treated with Aβ at concentrations closer to physiological levels, evidence for apoptosis is scarce; however, Aβ at physiological concentrations might render neurons more sensitive to cell stress. Intracellular Aβ1-42 exposure (as little as 1 picomolar) is, in contrast, toxic to human cortical neurons, through de novo protein synthesis, like Bax, p53, and caspases, suggesting cell death by apoptosis (Zhang et al, 2002).

Over-expression and intracellular accumulation of APP activates caspases, which themselves promote Aβ formation: increased production of Aβ may be therefore a consequence of neuronal apoptosis (Nikolaev et al, 2009).

Presenilin (PSEN) proteins can influence mitochondrial regulation of apoptosis and they are also substrates for caspase-3. However, over-expression of human wild-type or mutant PSEN1 or PSEN2 does not enhance apoptosis in neurons (Burstaěn et al, 1998; Gamliel et al, 2003).

Other work indicates that the presenilin-1 mutation sensitizes neurons to DNA damage-induced apoptosis (Chan et al, 2002).

More cell culture work therefore needs to be done on the basic mechanisms of human neurodegeneration and on Aβ neurotoxicity mechanisms, under basal conditions and in the presence of familial AD-related and tau gene mutations.

2.3.2. ANIMAL MODELS OF PARKINSON’S DISEASE

Information gleaned from molecular genetic studies of human genes linked to familial PD drives experimental work on the generation of animal and cell models of PD. Parkin null mice (α-Syn Tg Mice) appear to have a normal lifespan, do not develop any major neurological
abnormalities, and have no loss of midbrain dopaminergic neurons and no formation of inclusions (Goldberg et al, 2003). However, these mice exhibit evidence of dopaminergic presynaptic dysfunction in striatum and possible deficits in behavioral tests indicative of nigrostriatal dysfunction although this finding has not been confirmed in another mouse line (Perez et al, 2005). Parkin deficient-mice have decrease of proteins involved in mitochondrial oxidative phosphorylation and oxidative stress and reduced mitochondrial respiration in CNS, but no mitochondrial ultrastructural abnormalities (Palacino et al, 2004). In contrast, tg mice expressing the Parkin Q311X truncation mutation develop a progressive hypokinetic disorder, degeneration of SNc neurons, and loss of striatal dopamine (Lu et al, 2009). Thus, Parkin could be important for maintenance of mitochondrial function or mitochondrial turnover.

Similar findings were exhibited by mice with null mutations in DJ-1 (Goldberg et al, 2005). Several tg mouse lines have been made using different promoters to drive expression of human full-length wild-type or mutant α-Syn; it is noteworthy that there have been no reports of robust dopamine SNc neuron degeneration in full-length α-Syn tg mice, while mice expressing a truncation mutant of human α-Syn show a development-related loss of SNc neurons (Wakamatsu et al, 2008). Cell death mechanisms or thresholds for cell death activation in human and mouse brain dopamine neurons might differ.

Despite the absence of prominent changes in the SNc, tg α-Syn mice do develop robust cell death and neuronal loss in other regions of brain and in spinal cord (Martin et al, 2006). This interesting study about a tg mouse model of PD (mutant α-Syn A53T), actually resulted in a new model of Amyotrophic Lateral Sclerosis (ALS), thus providing insight into the selective vulnerability of motor neurons in age-related disorders and the possible roles of α Syn in synaptic maintenance and diseases of long-axon neurons.

3. FROM BENCH TO BED:
NEURODEGENERATIVE DISEASES

3.1. ALZHEIMER’S DISEASE

3.1.1. DEFINITION AND EPIDEMIOLOGY

Alzheimer's Disease is the most common cause of dementia. The fourth edition, text revised version of the DSM (DSM-IV-TR) published in 2000, identifies symptoms as criteria that must be met for a patient to be diagnosed with dementia: significant weakening of memory with
regard to learning new information as well as recalling previously learned one, accompanied by at least one of the following disturbances: aphasia, apraxia, agnosia, difficulties on executive functioning.

DSM-IV-TR also specifies that these disturbances must be severe enough to cause impairment in social and occupational functioning, and must represent a decline from a previously higher level of functioning.

In addition to cognitive impairment, symptoms of dementia may also include personality changes and emotional instability.

Finally, the diagnosis of dementia should not be made if the cognitive deficits occur exclusively during the course of a delirium.

In Italy dementia prevalence in individuals aged 65 and older is around 5-6%, increasing to 40% in people over 85 years (Ravaglia et al, 2008; Berry et al, 2005). Risk of dementia increases exponentially with age, and incidence doubles approximately every five years, from 0.2-0.8% in individuals aged 65-69 years to more than 5% in people over 80 years of age (Graham et al, 2008).

Alzheimer’s Disease accounts for 50-70% of all cases of dementia, followed by 20-30% of vascular/AD with cerebrovascular disease (Jellinger et al, 2008), Lewy Bodies Dementia (around 20%) (McKeith et al, 2007), frontotemporal spectrum dementias (10%) and secondary potentially reversible ones (10%) (Knopman et al, 2006; Di Carlo et al, 2002).

Among risk factors for dementia, age, familiarity and education are the more relevant ones (McCullagh et al, 2001). Literature data show that more than 85% of dementia onset happens after 65 years of age (McCullagh et al, 2001).

Familiarity for AD confers a 3-4 fold increased of developing the disease in first degrees relatives, if compared to general population risk. A family history of dementia may stand for the presence of genetic mutations or exposition to the same environmental risk factors interacting with genetic predisposition.

According to prevalence studies (Sando et al, 2008), education seems to be a protective factor against dementia. Several possible explanations have been proposed:

- an increase of synaptic plasticity directly proportional to education level
- a higher prevalence of vascular dementia risk factors (smoke, hypertension, dislipidemia) observed in subjects with lower education level (Caamaño-Isorna et al, 2006)
- high education does not protect against dementia development, but only mitigates disease impact on clinical expression (Brayne et al, 2010)

Among other possible risk factors, results are often contradictory.

Smoke is a known risk factor for stroke and therefore for vascular dementia, but its role on AD pathogenesis is less clear: while it seems to have a protective effect against AD mediated by up-regulation of nicotinic receptors (Sabbagh et al, 2002), more recent studies point to its
contribution to AD development, as it is related to cognitive and memory decline, as well as cerebral atrophy (Cataldo et al, 2010; Brody et al, 2004).

Another possible dementia risk factor is toxic exposure (glues, pesticides, solvents, aluminum in water) (Solfrizzi et al, 2006), while some drugs seem to have a neuroprotective effect, possibly mediated by an increase in choline transpherase activity in the case of estrogens (Pike et al, 2009), or related to inflammation inhibition for non steroids anti-inflammatory drugs (NSAIDS) (Etminan et al, 2003).


3.1.2. ANATOMOPATHOLOGY

The most striking macroscopic aspect of an AD brain is loss of weight and volume, which reflects global and usually symmetrical cortical atrophy, characterized by widening of sulci and Silvian fissure, and ventricular dilatation (Figure 2).

Atrophy mostly involves temporo-parietal lobes, with greater loss of hippocampal neurons, more prominent in entorinal cortex (Scahill et al, 2002), and sparing of primary motor cortex. Cortical thinning (usually around 10%) is the result of loss of neurons as well as of dendrites and dendritic spines, associated with astrocytes proliferation.

Brain stem degeneration is also remarkable in dementia patients, involving selective nuclei such as Meynert Nucleus Basalis (cholinergic input to cortex), Raphe serotoninergic Nucleus, adrenergic neurons of Locus Ceruleus and Substantia Nigra.

Microscopic hallmarks of AD are senile neuritic plaques and neurofibrillary tangles, which are necessary but not specific for AD diagnosis, as they are often present in healthy elderly (Figure 2).

Neuritic plaques are round aggregates, characterized by a central core of beta-amyloid (\(\text{A}_\beta\)), proteoglycans, ApoE, antichymotrypsin and several other proteins, surrounded by macrophages, microglia and dystrophic axons.

Neurofibrillary tangles are groups of helix filaments, mostly made of hyperphosphorylated tau protein, that surround cortical neurons nuclei.

Another typical aspect of AD brain is amyloid angiopathy, caused by amyloid deposition and consequent thickening of meningeal and cortical arteries wall (Robbins et al, 2006).

Definite AD diagnosis is made according to anatomopathological findings (NIA-Reagan et al, 1997).
3.1.3. PATHOGENESIS

From a molecular point of view, cerebral Aβ deposition is the crucial event in AD pathogenesis.

The so-called “amyloid hypothesis” postulates that the abnormal production of Aβ is the initial step in triggering the pathophysiological cascade that eventually leads to AD.

Aβ is a non-soluble protein made of 40-42 amino acids, deriving from proteolitic digestion of amyloid precursor protein (APP). This is a transmembrane protein with neurotrophic/neuroprotective function, and cleavage sites for α, β and γ-secretase enzymes. Aβ is generated from a sequential cleavage of APP operated by β and γ-secretase (Figure 3). The first one, called BACE-1, is an aspartyl protease, codified by a gene on chromosome 11, whose increased activity plays a fundamental role on Aβ accumulation and deposition (Vassar et al, 2009). Conditions like hypoxia, oxidative stress as well as inflammation also activate transcription factors whose action on BACE-1 gene promoter causes increased β-secretase expression (Vassar et al, 2008).

γ-secretase is an enzymatic complex of neuron plasmatic membrane and endoplasmic reticulum, composed by several proteins such as presenilins PSEN1 and PSEN2, nicastrin, and α1-antichimotripsyn; APP and Notch protein are among γ-secretase substrates.
Aβ formation starts from APP N-terminal cleavage operated by BACE-1, thus generating a C-terminal fragment attached to the membrane and a soluble fragment called sAPP.

γ-secretase cleavage on C-terminal then creates the APP intracellular domain peptide (AID) and Aβ peptides. As γ-secretase cleavage is not precise, most of those peptides have 40 aminoacids (Aβ 40) while a small portion - the toxic one - has 42 aminoacids (Aβ 42) (Cole et al, 2007).

Figure 3: sequential β e γ-secretase contribution to Aβ formation

Aβ rapidly folds in β sheets and aggregates, becoming neurotoxic through several mechanisms: astrocytes and microglia activation, stimulation of inflammatory response mediated by cytokines and oxidative stress, circulation impairment due to Aβ deposition on vessel walls, tau hyperphosphorylation (Iqbal et al, 2005).

Tau is a glycoprotein that promotes microtubules assembling and stabilization, thanks to its phosphatase groups. Tau hyperphosphorylation observed in AD is mediated by Aβ activated kinases and causes a loss of its physiological function as well as a toxic gain of function. The latest leads to normal tau sequestration and microtubules disruption, due to hyperphosphorylated tau aggregation in dimers (Figure 4).
Figure 4: Cascade of tau hyperphosphorylation and NFT formation

Neurofibrillary tangles, whose main component is hyperphosphorylated tau, lead to neuronal death and alteration of axonal transport, with consequent neurotransmission damage and dying back phenomena (Iqbal et al, 2005 and 2009).

3.1.4. GENETIC ASPECTS

Genetically, AD is usually divided into two forms: (1) familial cases with Mendelian inheritance of predominantly early-onset (early-onset familial AD, EOFAD), and (2) so-called “sporadic” cases with no apparent familial aggregation and usually of later onset age (late-onset AD, LOAD). EOFAD accounts for 2% of cases and is usually characterized by early onset (<60 years) and autosomal dominant transmission, LOAD represent 98% of AD cases.

It needs to be emphasized that this traditional dichotomization is overly simplistic as there are cases of early-onset AD without evidence of Mendelian transmission while, conversely, “sporadic” LOAD is frequently observed with a strong familial clustering, and up to 60%-80% of this form is genetically determined (Gatz et al, 2006).

While EOFAD is caused by rare and highly penetrant mutations in three genes (see below), the genetics of LOAD is more complex. Current thinking posits that susceptibility for LOAD is conferred by numerous genetic risk factors of relatively high frequency but low penetrance and therefore small effect size (Bertram et al, 2010).

First progress on genetics of AD was afforded by studying large, multigenerational pedigrees suffering from early-onset familial AD. Assessing coinheredence of specific genetic markers in genetic linkage analyses (see later on in the text) provided a rough estimate of the most
likely location of the underlying disease gene, which was subsequently identified by means of “positional cloning” (a more or less systematic mutational screening of DNA segments close to the linkage peak).

With this approach, three genes responsible for familial AD were identified, with more than 200 distinct disease-causing mutations known so far (AD & FTD Mutation Database, http://www.molgen.ua.ac.be/admutations/). The discovery of these genes had significantly contributed to understanding of pathogenetic mechanisms underlying neurodegeneration in AD, in particular the “amyloid hypothesis”.

APP (Amyloid Precursor Protein) gene is the first discovered, localized on chromosome 21. APP gene mutations compromise APP metabolism and increase toxic amyloid fragments production (Basun et al, 2008). This gene is also involved in AD development in subjects with Down’s Syndrome: chromosome 21 trisomy implies excessive production of brain amyloid (Rovelet-Lecrux et al, 2006) thus causing dementia usually after the age of 40 and anatomopathological findings typical of AD.

After APP, two other genes have been discovered: they codify for presenilin proteins 1 and 2 (PSEN1 and 2) and are located on chromosome 14 and 1 respectively. PSEN1 product is a protein called S182, whose mutations increase BACE-1 expression and activity, and therefore Aβ42 production. These mutations are responsible for earlier AD onset than PSEN2 ones, which involve a protein called SMT2 (Levy-Lahad et al, 1995). Both presenilins belong to γ-secretase complex, specifically to its proteolytic portion (Helig et al, 2010).

These three EOFAD genes explain a large proportion - but not all - of the Mendelian forms of AD, making it likely that additional EOFAD-causing genes exist.

As previously told, 98% of AD cases are sporadic and multifactorial; that means that pathogenesis is related to synergic interaction between several predisposition genes, located in independent loci, and environmental factors.

Several “candidate genes” assessed for genetic association with AD have been so far identified. Among them, the first and so far probably most important is APOE gene (apolipoprotein E), involved in both familiar and sporadic late onset AD. It is located on chromosome 19q13 and codifies for a protein expressed in several organs, with the highest expression in the liver, followed by the brain (mainly by astrocytes and to some extent microglia, at much lower levels by neurons). APOE functions as a ligand in receptor-mediated endocytosis of lipoprotein particles. The three allelic forms of APOE (ε2, ε3, ε4) differ from each other for amino acid substitutions, conferring physical and biochemical specific properties that influence APOE activity in amyloid clearance. APOE ε4 is related to insufficient Aβ degradation, thus facilitating its deposition (Kim et al, 2009) and being therefore responsible for a dose-dependent ~4-fold increase in AD risk if compared to noncarriers. This continues to be the lead association finding of LOAD. In contrast to ε4, the rarer ε2 allele appears to have a “protective” effects.
The nonspecific nature of the APOE-AD association (APOE seems to be involved also in PD, multiple sclerosis, macular degeneration, cardio and cerebrovascular diseases, etc) has made researchers hypothesize a role for other genes/proteins close to APOE in AD predisposition.

In the last 30 years, the candidate gene approach lead to the investigation of nearly 700 candidate AD genes, but only few show significant risk effects; among them ACE, ADAM10, IL8, MTHFR, Nicastrin, SORL1, etc. (see the AlzGene Database).

As one of the main limitations of the candidate gene approach is its focus on a priori functional and/or positional hypothesis, the advent of microarray technology has allowed genetics research to assess several hundreds of thousands of single-nucleotide polymorphisms (SNPs) in one experiment.

Several genome-wide association studies (GWAS) have been performed in AD to date, but only a few were reported to show genome-wide significance; in many others independent replication of findings was inconsistent (Bertram et al, 2010).

In 2009, two large association studies (Lambert et al, 2009; Harold et al, 2009) have identified three novel AD genes, i.e. CLU (clusterin or apolipoprotein J), CR1 (complement component receptor 1), and PICALM (phosphatidylinositol-binding clathrin assembly protein). All three of these loci have received support from independent follow-up studies (Carrasquillo et al, 2010).

CLU (Clusterin) gene on chromosome 8 codifies for a protein called Clusterin, a 75 kDa chaperone molecule expressed in all tissues, including the CNS; it seems to have a neuroprotective function through Aβ clearance and be involved in Aβ fibrillization, regulation of brain cholesterol and lipid metabolism, and inhibition of neuronal apoptosis.

PICALM (phosphatidylinositol-binding clathrin assembly protein) gene on chromosome 11 codifies for a Clatrin protein, involved in synaptic transmission, endocytosis, removal of apoptotic cells, and possibly in Aβ clearance.

CR1 (complement receptor-1) gene on chromosome 1 codifies for the main receptor of the complement C3b protein, a key inflammatory protein activated in AD. In vitro and in vivo experiments suggest that complement activation can protect against Aβ-induced neurotoxicity and may reduce the accumulation/promote the clearance of amyloid.

In 2010 Zou et al. identified IDE (insulin-degrading enzyme) gene, on chromosome 10, as a LOAD gene. It codifies for a metallopeptidase located in cytosol and its structures, responsible for degradation of small proteins like insulin, glucagon and Aβ. They performed a pilot study using cerebellar gene expression levels of 12 LOAD candidate genes and their cis-single nucleotide polymorphism (SNP) genotypes extracted from their LOAD GWAS. Most of these messenger RNAs (mRNAs) encoded proteins that are likely to be involved in neurodegeneration. Authors also suggested that the use of expression levels as “endophenotypes” in genome-wide association
studies may provide a powerful approach for the identification of disease susceptibility alleles (Zou et al, 2010).

Two further GWAS were published in 2010, suggesting the existence of three additional AD susceptibility loci.

The first study resulted from a large collaboration (Seshadri et al., 2010); in addition to replicating the association between CLU and PICALM, authors found two potential additional AD risk factors: **BIN1** (*bridging integrator*1) and **EXOC3L2** (*exocyst complex component 3-like* 2). Combining all available data, both genes display highly significant association with AD risk on AlzGene, with p values around $3.0 \times 10^{-10}$ and $2.1 \times 10^{-10}$, respectively.

**BIN1** (also known as amphiphysin II) encodes several isoforms of an adaptor protein involved in receptor-mediated endocytosis which could have an effect on Aβ production and/or the clearance of Aβ from the brain.

The biological function of the protein encoded by **EXOC3L2** remains largely elusive, but is also noteworthy that the associated SNP maps only ~300 kb distal to the APOE region, so it remains to be seen whether these two regions are genetically or functionally related (maybe there’s only a “tagging” of the association with APOE and does not actually represent a novel AD locus in its own right).

The latest addition to GWAS-derived putative LOAD loci is **MTHFD1L** (*methylenetetrahydrofolate dehydrogenase 1-like*), reported to show genomewide significant association with AD risk in ~5,000 individuals (Naj et al, 2010). In contrast to most other AD GWAS findings, the risk allele appears to confer allelic ORs ~2, which means nearly doubling the risk for AD in carriers of the minor allele.
3.1.5. CLINICAL ASPECTS

Alzheimer’s disease has an insidious onset, and a progressive course that usually leads to death in a decade or so.

This course can be educationally divided into three phases, with a first period characterized by symptoms onset and slow progression, followed by a stage of stabilized symptoms, and a third terminal one; these stages reflect the progressive involvement of different cortical areas, starting from hippocampus and mesial temporal areas, and progressing towards temporo-parietal structures.

Exact clinical AD onset is really difficult to describe, as there is a great inter-individual variability of symptoms, which are often quite vague and underestimated.

Among the most frequent symptoms at presentation, temporal disorientation and an amnestic syndrome are very typical, and usually point to a suspicion of Alzheimer’s Disease. Considering memory deficit, short term memory is the most compromised, characterized by a difficulty in remembering/acquiring new information. In the first stages of the disease, long term semantic and biographical memories are usually spared.
Another quite common symptom at onset is aphasia nomenum, which means difficulty in naming people and objects, consequently leading to the use of generic “passé partout” words like “that”, “this”, “thing”, etc.

Patients with an early involvement of parietal lobes, often develop since the beginning visuospatial deficits, that sometimes are revealed only with neuropsychological test (e.g. figure copy); they usually have constructive apraxia, at the beginning for three-dimensional objects, then for two-dimensional ones.

Attention and concentration difficulties are also quite common, even in the first phase of the disease; sometimes relatives observe subtle personality change, as well as mood fluctuation, with increased anxiety and depression, irritability and anger, or apathy and abulia. Usually in this first phase there are no motor complains and/or pathological findings on neurological examinations.

Obviously this is only the description of a typical AD patient, as there are many atypical forms resembling other subtypes of dementia, such as behavioural variant of frontotemporal dementia or corticobasal degeneration, or starting with prominent parietal involvement, such as primary progressive aphasia or posterior cortical atrophy.

After a variable period of a few months/more than a year, progression of symptoms becomes more evident and a definite clinical picture of the disease appears. The patient is usually disoriented in time but also in place, he can get lost, forget or does not recognize knows places; usually in a more advanced phase, disorientation inside his own house happens.

Memory becomes more and more compromised, involving not only recent events, but also biographical ones and even semantic knowledge (Taylor et al, 2007), leading to a global amnestic syndrome.

Language disruption progressively leads from anomas to severe communications difficulties, due to a more and more telegraphic and “empty” verbal production, with anomas, semantic/phonemic paraphasias, and neologisms, together with an increasing difficulty in comprehension due to loss of semantic knowledge and memory problems. Agraphia and alexia are almost always present (Hebert et al, 2000).

All the other cortical functions are progressively compromised, with development of agnosia, both for objects and people, as well as constructive and ideomotor apraxia.

In this phase, behavioral symptoms, as well as delusions and hallucinations usually accompany cognitive ones, often becoming the most relevant aspects for quality of life of both patient and relatives/caregivers (Rozzini et al, 2008).

Motor signs like bradikinesia and extrapyramidal syndrome complete the clinical picture.

This phase of the disease usually lasts from 3 to 8 years, and is followed by the terminal one, when the patient cognitive profile is completely disrupted, usually characterized by mutism, rigidity, incontinence, complete dependence also for basic activities (Lechowski et al, 2009).
Automatisms and release reflexes like grasping, sucking reflex, as well as myoclonus and epileptic seizures usually appear in this phase. As a consequence of loss of autonomy, the patient evolution is towards cachexia and death in 6-12 months.

3.1.6. DIAGNOSIS

Criteria that must be met for a patient to be diagnosed with dementia are significant weakening of memory with regard to learning new information as well as recalling previously learned one, accompanied by at least one of the following disturbances: aphasia, apraxia, agnosia, difficulties on executive functioning.

DSM-IV-TR (2000) also specifies that these disturbances must be severe enough to cause impairment in social and occupational functioning, and must represent a decline from a previously higher level of functioning.

For diagnostic and therapeutical purposes, identification of the possible cause of dementia is needed.

Detailed patient history, complete clinical exam, neuropsychological tests as well as laboratory and radiological instruments are combined to reach this goal.

History wise, particular attention must be put on family history of dementia, psychiatric diseases and/or sistemic diseases that can cause cognitive decline. Mental retardation, education, nutrition, habits (e.g.drugs, smoke, substance abuse) are all aspects of personal history which must be addressed to.

Accurate evaluation of past medical history, with particular attention to head traumas, neuropsychiatric, endocrinal, cerebro/cardiovascular diseases and vascular risk factors such as hypertension, diabetes, atrial fibrillation, dislipidemia, can help distinguishing secondary dementias from neurodegenerative ones. Last but not least, history of present illness allows to go into type of onset and evolution of the cognitive decline.

Considering clinical examination, it must start from the initial approach to the patient that often allows to identify attention, memory, orientation, and language deficits, as well as mood and behavioral aspects.

General conditions and signs of systemic diseases possibly related to a dementia syndrome must be looked at, but is the neurological examination an essential part of the patient’s evaluation, through the observation of focal neurological signs, posture, walking patter, reflexes and muscle tone.

The patient’s picture must be completed by a neuropsychological standardized evaluation of all cognitive domains. Neuropsychological tests are useful methods to be used also for disease
progression monitoring. Is not in the interest of this thesis to describe which tests to be used in the
assessment of a patient with dementia.

However, among them, Mini Mental State Examination (MMSE) and Ten Point Clock Test
(TPCT) are commonly applied for screening purposes.

MMSE (Folstein et al, 1975) is a short test widely used in clinical practice to evaluate
cognitive functions, especially in elderly people; it is rapid and easy to be administered, and quite
reliable in grading and following dementia progression. It comprises specific tests for different
cognitive domains, and a score < 24/30 has a sensibility of 80-90% and a specificity of 80% in
distinguishing people with and without cognitive decline (Fountoulakis et al, 2000). A score
between 25 and 30 means absence of cognitive decline, from 19 to 24 mild dementia, from 11 to
18 moderate dementia, severe dementia if below 10 (Ward et al, 2002).

TPCT (Manos et al, 1994) is a short and easily understandable test, like MMSE; it
measures visuospatial and executive functions, abstract reasoning and symbolic representation
(Peters et al, 2008). This test correctly classifies 77% of AD patients, 89% of individuals with
multinfarct and mixed dementia, and 78% of healthy subjects (Agrell et al, 1998).

There are many other tests measuring global or specific cognitive functions, but also mood
(e.g. Geriatric Depression Scale, GDS) (Craen et al, 2003), behavior (Neuropsychiatric Inventory,
NPI) (Commings et al, 1994), activities of daily living (e.g. Instrumental Activities of Daily Living
Scale, IADL) (Jefferson et al, 2006), severity of dementia (e.g. Clinical Dementia Rating, CDR)
(Perneczky et al, 2006).

Clinical and neuropsychological evaluation must be completed by laboratory (e.g. renal
and liver function, glucose, thyroid hormones, folate and B12 vitamin) and neuroimaging exams.
Cerebral CT and MRI are first level tools to be used not only for the exclusion of possible reversible
causes of dementia (e.g. normal pressure hydrocephalus, tumors, subdural hematoma), but also
for atrophy and vascular burden evaluation.

Functional imaging, such as Single Photon Emission Tomography (SPECT) and Proton
Emission Tomography (PET), are second level exams reflecting cerebral perfusion and
metabolism, and commonly used when clinical picture and atrophy characteristics are not
sufficiently informative (Ito et al, 2006).

Among diagnostic criteria for Alzheimer’s Disease, NINCDS-ADRDA (National Institute and
Communicative Disorders and Stroke; Alzheimer's disease and Related Disorders Association
Work Group Criteria) have a high positive predictive value (McKhann et al, 1994; Dubois et al,
2007).
I. The criteria for the clinical diagnosis of PROBABLY Alzheimer's disease are illustrated in Panel B (Dubois et al, 2007):

### Panel 2: Diagnostic criteria for AD

**Probable AD:** A plus one or more supportive features B, C, D, or E

**Core diagnostic criteria**
- Presence of an early and significant episodic memory impairment that includes the following features:
  - Gradual and progressive change in memory function reported by patients or informants over more than 6 months
  - Objective evidence of significantly impaired episodic memory on testing that generally consists of recall deficit that does not improve significantly or does not normalise with cueing or recognition testing and after effective encoding of information has been previously controlled
  - The episodic memory impairment can be isolated or associated with other cognitive changes at the onset of AD or as AD advances

**Supportive features**
- Presence of medial temporal lobe atrophy
  - Volume loss of hippocampi, entorhinal cortex, amygdala evidenced on MRI with qualitative ratings using visual scoring (referenced to well-characterised population with age norms) or quantitative volumetry of regions of interest (referenced to well-characterised population with age norms)
- Abnormal cerebrospinal fluid biomarker
  - Low amyloid $\beta_{40}$ concentrations, increased total tau concentrations, or increased phospho-tau concentrations, or combinations of the three
  - Other well-validated markers to be discovered in the future
- Specific pattern on functional neuroimaging with PET
  - Reduced glucose metabolism in bilateral temporal parietal regions
  - Other well-validated ligands, including those that foreseeably will emerge such as Pittsburgh compound B or FDDNP
- Proven AD autosomal dominant mutation within the immediate family

II. The criteria for the clinical diagnosis of POSSIBLE Alzheimer's disease include:

A. Dementia syndrome in the absence of other neurologic, psychiatric, or systemic disorder

OR

B. Presence of a second systemic or brain disorder sufficient to produce dementia, which is not considered to be the primary cause of the dementia

III. Exclusion criteria for a diagnosis of Probable or Possible AD unlikely or uncertain are listed in the panel below (Dubois et al, 2007):
IV. AD is considered **DEFINITIVE** if the following are present:

A. Both clinical and histopathological (brain biopsy or autopsy) evidence of the disease, as required by NIA-Reagan (NIA-Reagan, 1997) criteria for the post-mortem diagnosis of AD; criteria must both be present

B. Both clinical and genetic evidence (mutation on chromosome 1, 14, or 21) of AD; criteria must both be present

According to this, clinical diagnosis is just a probability one, where history, neurological, neuropsychological, laboratory and neuroimaging data contribute to a diagnostic accuracy of 85-90% (Cassano, 2006).

Among criteria for a diagnosis of vascular dementia (Pohjasvaara et al, 2000), the NINDS-AIREN ones (National Institute of Neurological Disorders and Stroke; Association Internationale pour la Recherche et l’Enseignement en Neurosciences) are very commonly used (Roman et al, 1993).

I. The criteria for the clinical diagnosis of **probable** vascular dementia include all of the following:

1. Dementia defined by cognitive decline from a previously higher level of functioning and manifested by impairment of memory and of two or more cognitive domains (orientation, attention, language, visuospatial functions, executive functions, motor control, and praxis), preferable established by clinical examination and documented by neuropsychological testing; deficits should be severe enough to interfere with activities of daily living not due to physical effects of stroke alone.
Exclusion criteria: cases with disturbance of consciousness, delirium, psychosis, severe aphasia, or major sensorimotor impairment precluding neuropsychological testing. Also excluded are systemic disorders or other brain diseases (such as AD) that in and of themselves could account for deficits in memory and cognition.

2. Cerebrovascular disease (CVD), defined by the presence of focal signs on neurologic examination, such as hemiparesis, lower facial weakness, Babinski sign, sensory deficit, hemianopsia, and dysarthria consistent with stroke (with or without history of stroke), and evidence of relevant CVD by brain imaging (CT or MRI) including multiple large vessel infarcts or a single strategically placed infarct (angular gyrus, thalamus, basal forebrain, or PCA or ACA territories), as well as multiple basal ganglia and white matter lacunes, or extensive periventricular white matter lesions, or combinations of them.

3. A relationship between the above two disorders, manifested or inferred by the presence of one or more of the following: (a) onset of dementia within 3 months following a recognized stroke; (b) abrupt deterioration in cognitive functions; or fluctuating, stepwise progression of cognitive deficits.

II. Clinical features consistent with the diagnosis of probable vascular dementia include the following:

(a) Early presence of gait disturbance (small-step gait or marche a petits pas, or magnetic, apraxic-ataxic or parkinsonian gait); (b) history of unsteadiness and frequent, unprovoked falls; (c) early urinary frequency, urgency, and other urinary symptoms not explained by urologic disease; (d) pseudobulbar palsy; and (e) personality and mood changes, abulia, depression, emotional incontinence, or other subcortical deficits including psychomotor retardation and abnormal executive function.

III. Features that make the diagnosis of vascular dementia uncertain or unlikely include (a) early onset of memory deficit and progressive worsening of memory deficit and progressive worsening of memory and other cognitive functions such as language (transcortical sensory aphasia), motor skills (apraxia), and perception (agnosia), in the absence of corresponding focal lesions on brain imaging; (b) absence of focal neurological signs, other than cognitive disturbance; and (c) absence of cerebrovascular lesions on brain CT or MRI.

IV. Clinical diagnosis of possible vascular dementia may be made in the presence of dementia (section I-1) with focal neurologic signs in patients in whom brain imaging studies to confirm
definite CVD are missing; or in the absence of clear temporal relationship between dementia and stroke; or in patients with subtle onset and variable course (plateau or improvement) of cognitive deficits and evidence of relevant CVD.

V. Criteria for diagnosis of definite vascular dementia are (a) clinical criteria for probable vascular dementia; (b) histopathologic evidence of CVD obtained from biopsy or autopsy; (c) absence of neurofibrillary tangles and neuritic plaques exceeding those expected for age; and (d) absence of other clinical or pathological disorder capable of producing dementia.

VI. Classification of vascular dementia for research purposes may be made on the basis of clinical, radiologic, and neuropathologic features, for subcategories or defined conditions such as cortical vascular dementia, subcortical vascular dementia, Binswanger Disease, and thalamic dementia. The term "AD with CVD" should be reserved to classify patients fulfilling the clinical criteria for possible AD and who also present clinical or brain imaging evidence of relevant CVD. Traditionally, these patients have been included with VaD in epidemiologic studies. The term "mixed dementia," used hitherto, should be avoided.

3.1.7. THERAPY

Dementia treatment consists of a double approach.

The non-pharmacological one is based on rehabilitation techniques and care of cognitive, affective, relational and behavioral aspects.

The pharmacological approach aims at intervening on both cognitive and behavioral symptoms, and hopefully at delaying disease progression. Medication so far available are only symptomatic, as there is no drug that can prevent, heal or slow down disease course.

Acetylcholinesterase Inhibitors (AChEI) inhibits the cholinesterase enzyme from breaking down acetylcholine, increasing both level and duration of action of the neurotransmitter acetylcholine.

Several studies demonstrated the efficacy of AChEI in treating Alzheimer’s Disease, characterized by cholinergic deficit.

Their effect is hopefully supposed be seen on both cognitive and behavioral symptoms, manifesting as an improvement of memory, language and concentration abilities, as well as increased motivation and decreased aggressiveness. Although, there is a wide interindividual variability, and usually after a mild improvement in the first period of treatment (around one year), responsiveness to this drugs progressively decreases. (Neugroschl et al, 2010; Hansen et al, 2008).
Among AChEI, Donepezil, Rivastigmine and Galantamine are those most frequently used (Hansen et al, 2008). Some clinical trials also demonstrated efficacy of Donepezil and Galantamine in vascular dementia treatment, where they observed an improvement of cognitive and behavioral profile, as well as of ability in activities of daily living (Demaerschalk et al, 2007). However, other studies found a clinically not significant cognitive benefit, and insufficient proofs to support their widespread use in vascular dementia (Kavirajan et al, 2007).

In this subtype of dementia, great part of therapeutical action is towards prevention of new ischemic events, through both proper control of risk factors and administration of blood-platelet antiaggregants (Aspirin, Ticlopidine, Clopidogrel) or antiacoagulants (Eparin, Warfarin, Acenocoumarol).

Another drug, born for AD treatment, seems to have mild beneficial effect also on vascular dementia: Memantine acts on the glutamatergic system by blocking N-Metil-D-aspartate (NMDA) glutamate receptors; it thus inhibits neurotoxic excitatory action of Glutamate without interfering with its physiologic role on memory and learning (McShane et al, 2006).

A part from cognition, Memantine is also known to induce an improvement on behavioral aspect of dementia, in particular its use seems to be related to a decrease in aggressiveness, irritability and eating disorders (Cummins et al, 2006).

There’s another category of medications widely used in dementia, called nootropics, which are supposed to improve cognition by directly acting on cerebral metabolism (Piracetam, Oxiracetam) or through antioxidant effect (Selegiline, Vitamin E, Ginko Biloba) (Leuner et al, 2010; Pratico’ et al, 2008; Tsolaki et al, 2001).

Behavioral and psychological symptoms of dementia (BPSD) like agitation, wandering, aggressiveness, irritability, hallucination/illusion, disinhibition, euphoria, depression, are often the most challenging to be treated, and in the same time the most relevant for quality of life of both patient and caregiver.

Safety of antipsychotics in elderly patients with dementia has been hardly debated; the new generation ones, called atypical, are the best option for short-term (6–12 weeks) treatment of BPSD that is severe and persistent, but serious cardiovascular adverse events are a major contraindication to long-term therapy and their use (Gareri et al, 2010). For this reason, the presence of cardiovascular diseases, QTc interval on electrocardiogram, electrolytic imbalances, familiar history for torsades des pointes, concomitant treatments and use of drugs able to lengthen QTc have to be closely taken into account before prescribing them (Jeste et al, 2008).

Several studies demonstrated a beneficial effect of physical exercise on dementia symptoms, with improvement of cognition and quality of sleep, decreased behavioral symptoms and less need of antipsychotics use (Lautenschlagher et al, 2006; Geda et al, 2010).
3.2. PARKINSON’S DISEASE

3.2.1. DEFINITION AND EPIDEMIOLOGY

Parkinson’s Disease was firstly described in 1817 by James Parkinson in “An Essay of the Shaking Palsy”.

According to World Health Annual Report of 2004, neurodegenerative diseases affect more than 7% of worldwide population (450 million people) and among them PD is the second most frequent, preceded only by Alzheimer’s Disease.

Epidemiological data, mostly recorded in industrialized countries, indicate an annual incidence rate of 4.9 - 23.8 new cases every 100.000 inhabitants, with an increase after 65 years (Antonini et al, 2005). As incidence remained almost the same in the last 45 years, prevalence progressively increased due to improvement in treatment and rehabilitation techniques, and in line with general demographic trend. According to most of the studies, worldwide prevalence of PD is between 84 and 270 cases every 100.000 inhabitants; mean age of onset is 48-62 years, with modal distribution between 57 and 77 years (Antonini et al, 2005).

Baldereschi et al. in 2000 reported an average annual incidence rate of 326.3 (95% CI, 224.1 to 427.5) per 100,000 person-year in Italian population aged 65 to 84 years, adjusted to the 1992 Italian population, is Incidence rates increase with age in both men and women, with men having higher rates in every age group; age-adjusted relative risk in men compared with women was 2.13 (95% CI, 1.11 to 4.11).

Mortality after 10 years from diagnosis, which was 2.9% in pre-levodopa era, halved after the introduction of this drug. Mean survival still remains lower if compared to general population (Ishihara et al, 2007).

Since the 70’s, many prospective studies tried to identify possible risk factors for PD, with surprising results such as inverse correlation between cigarette smoking and PD or the absent influence of nutrition, alcohol and CNS infections on disease development. More recent studies found a robust correlation with some environmental factors such as pesticides, herbicides and heavy metals (Antonini et al, 2005).

A part from monogenic forms (accounting for 5-10% of total PD), is now widely accepted that PD belongs to complex or multifactorial diseases, where environment influence acting upon a genetically contributes to disease phenotype.
3.2.2 ANATOMOPATHOLOGY

Physiological age-related dopamine depletion is around 6-8% every 10 years (Hornykiewicz, 1985), implying a 40-50% reduction in healthy subjects after the age of 60; in PD patients, data from post-mortem studies seem to indicate first symptoms appearance at 70-80% of nigral dopamine lost (Bernheimer et al, 1973), as before that threshold increased metabolic processes as well as compensation phenomena happen in residual neuronal circuitries.

Anatomopathological studies revealed also cellular depigmentation (Figure 5) with extraneural and inside phagocytes melanin deposition, as well as eosinophilic intracytoplasmic inclusions called Lewy Bodies in surviving neurons.

These findings, prominent in substantia nigra (SN), are widely distributed in different CNS areas, like locus coeruleus, dorsal motor nucleus of the vagus nerve, substantia innominata, intermediolateral cell column of the spinal chord and cerebral cortex.

![Figure 5: Comparison of SN in healthy subject (left) and PD patient (right); prominent depigmentation is pointed by arrows.](image)

Even if present in 80% on PD patients, Lewy Bodies (LB) are not pathognomonic of this disease, as they are found in Shy-Drager Disease, Multisystem Atrophy, Lewy Body Dementia, etc. but also in more than 5% of autopsy reports of normal subjects older than 65 years.

LBs are intracytoplasmic aggregations of proteins, fatty acids, sphingomyelin and polysaccharides, appearing at electron microscope as a central dense core, surrounded by a typical halo. Their principal component is α-synuclein, modified through several post-translational processes such as hyperphosphorylation, oxidation and ubiquitination.

Observation of premotor signs and symptoms of PD, typical of prodromal phase of the disease, lead to a revision of the neurodegenerative process progression.

Braak et al. in 2003 established a specific anatomopathological stadiation of PD, based of immunohistochemical detection of LB (Braak et al, 2003).
According to this model, neuropathological process would begin in motor nucleus of vagus, substantia reticularis and glossopharyngeal nucleus in oblongata medulla (stages I and II); disease progression would extend towards olfactory nucleus and then substantia nigra (stage III). Stages IV and V are characterized by presence of LB in basal forebrain/transentorinal cortex and associitative/prefrontal cortices respectively.

3.2.3. PATHOGENESIS

Even if PD pathogenesis is mainly related to degeneration of mesencephalic dopaminergic neurons of central and caudal portion of substantia nigra-pars compacta (SNc), and consequent reduction of striatal dopamine levels, nigrostriatal dopamine is not the only neurotransmitter involved.

In PD, the noradrenergic system, projecting from locus coeruleus to neocortex, amygdala and hippocampus, is characterized by a 50% reduction of noradrenalin, if compared to healthy controls (Scatton et al, 1983).

Serotonergic system is also impaired in PD patients, as demonstrated by lower levels of serotonin in striatum, substantia nigra and hippocampus (Bernheimer et al, 1973).

Considering cholinergic system, the balance dopamine/acetylcholine implies – as a consequence of dopamine depletion – an increase in excitatory cholinergic function of striate neurons. This aspect has been used in the past for therapeutical purposes.

Since the 80’s, strong evidences emerged in favor of a reduction of cholinergic levels in putamen, caudate nucleus, globus pallidus, but also in several cortical areas i.e. frontal, entorinal, cingulated and occipital cortices, as well as hippocampus.

Gabaergic transmission wise, its role in PD pathogenesis is still not clear and needs further studies, but a reduction of gabaergic activity of Luys subtalamic nucleus seems to emerge.

Summarizing, actual knowledge on PD pathogenesis is still limited, especially considering molecular mechanisms underlying nigrostriatal dopaminergic degeneration.

Common opinion is that they probably result from interaction between intracellular proteolysis, susceptibility towards oxidative stress as well as mitochondrial dysregulation, caused by environmental toxic exposition acting on genetically determined susceptibility: like AD, PD therefore represents a good model of multifactorial disease.
3.2.4. CLINICAL ASPECTS

Diagnosis of PD is still based on clinical aspects, as no biochemical and or specific neuroimaging marker is so far available.

Clinical diagnosis requires the detection on neurological examination of almost 2 out of 4 essential symptoms: tremor, rigidity, bradikinesia and postural instability.

Disease course is characterized by a great interindividual variability; Martilla and Rinne in 1990 reported that time elapsing between symptoms onset and terminal phase of the disease is usually around 8.5 years.

Prodromal phase of the disease is often characterized by aspecific symptoms, such as faticability, diffuse pain, depressive syndrome, hypoosmia and sleep disorders (Tolosa et al, 2009).

A 3-6 Hz distal resting tremor, usually in upper limbs, is the first complain in 50,6% of patients (Colosimo et Antonini, 2005), but in 20% of cases bradikinesia preceding tremor is noted by family members (Marsden e Fahr, 1981). Tremor is probably related to involvement of thalamo-cortical network, deriving from nigro-striatal and rubro-cerebellar circuits damage (Berardelli et al, 2003).

Bradikinesia, defined as a global reduction of speed, amplitude and rythm of movements, seems to derive from an underscaling of motor commands for internally generated movements. This explains the increased dependance of PD patients motor performance from external cues and reflects physiological role of basal ganglia in selecting and reinforcing cerebral activity during planning and execution of a voluntary movement (Berardelli et al, 2003).

Gait is typically compromised in PD patients, who exhibit shuffling gait with small steps, poverty of movements in the trunk and reduced, associated swing movements in the upper limbs. The initiation of locomotion is difficult and eventually the gait becomes arrested, "frozen" (Giladi et al, 1992), or an involuntary hastening in gait, propulsion (or festination) may occur. Falls and loss of balance are quite frequent, especially in the advanced stages of the disease, due to compromission of postural reflexes.

Other symptoms and signs, like hypomimia (“facies frigeè”), blink reduction, monotone and hypophonic speech, can be considered different expressions of bradikinesia.

Another cardinal symptom of PD is increased tone or “rigidity”, defined as a resistance to passive movement. In PD, two types of rigidity are classically recognized which may coexist: “leadpipe ” that means rigidity throughout the whole range of motion, and “cogwheel" that is a combination of rigidity and tremor which presents as a jerky resistance to passive movement.

The forth cardinal symptom of PD is postural instability, which is often the most disableng one as responsible of falls (Guttman et al, 2004) and usually less responsive to pharmacological treatments. It results from a combination of rigidity, bradikinesia and alteration of physiological postural reflexes, which allow to quickly respond to balance and motor scheme perturbations.
Spectrum of symptoms associated with PD also includes several other manifestations, often wrongly defined as "minor": depression in 20-50% of PD population (Lennox et al, 2002); anxiety (Evans et al, 1999) and panic attacks (Shulman et al, 2002) in 40%; hallucinations and other psychiatric symptoms in 16-37% of patients in advanced stages of the disease, often in presence of cognitive decline (Aarsland et al, 1999); apathy, probably symptomatic of cingulate cortex involvement (Shulman et al, 2002); sleep disorders, among whom REM Sleep-related Behavior Disorder (RBD), characterized by loss of muscular atonia during REM phase and presence of complex motor activity, often accompanied by aggressiveness and vocalizations (Onofri et al, 2002).

PD patients also have 2-3 fold increased risk of developing dementia, if compared to general population (Aarsland et al, 2001); dementia prevalence in PD patients older than 65 years is around 15-40% (Lennox et al, 2002).

Another important aspect of Parkinsonian clinical picture that is worth it to be mentioned is Autonomic Nervous System dysfunction: gastroenteric symptoms like dysphagia, delayed gastric emptying and constipation; orthostatic hypotension in 15% of patients (Martignoni et al, 1995); thermoregulation problems like abrupt and fluctuating sweating (Raudino et al, 2001).

In summary, the so called “non-motor” symptoms of PD are becoming a more and more important component of the parkinsonian syndrome, as they influence as much as motor problems the patient’s quality of life (Soh et al, 2010).

3.2.5. GENETICS AND GENOMICS

In the last decade, advances in genomic analysis opened new perspectives in knowledge of pathogenetical mechanisms underlying dopaminergic neurodegereration. PD like AD is considered a multifactorial or complex disease, where interaction among different independent susceptibility genes creates a predisposition for disease development. On that background, environment influence contributes to final phenotype.

Only a small percentage of PD cases (3-5%) are genetically determined, even if it can reach 77% in groups of patients selected by age of onset, familiarity and ethnicity.

Mendelian forms of PD are characterized by important heterogeneity, not only in genetic-molecular terms, but also considering age of onset, progression speed and histopathological findings (Bonifati et al, 2003).

At the same time, some monogenic forms exhibit a convergent phenotype, sometimes indistinguishable from idiopathic PD (Klein et al, 2007).

Chromosomic loci associated with familiar forms of PD are classified as “PARK” followed by a specific number. There are six autosomal dominant forms (PARK1-3-4-5-8-11), six recessive
forms (PARK2-6-7-9-14-15) and one X-linked (PARK-12), while transmission model of PARK10 e PARK13 has still to be defined (see table below; Martin, 2010).

### Mutant Genes Linked to Familial PD.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Inheritance</th>
<th>Gene</th>
<th>Protein Name/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1/4q21</td>
<td>autosomal dominant</td>
<td>α-syn</td>
<td>α-Syn/presynaptic maintenance?</td>
</tr>
<tr>
<td>PARK2/6q25.2-27</td>
<td>autosomal recessive</td>
<td>parkin</td>
<td>Parkin/ubiquitin E3 ligase</td>
</tr>
<tr>
<td>PARK3/p13</td>
<td>autosomal dominant</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>PARK4/4p15</td>
<td>autosomal dominant</td>
<td>α-syn</td>
<td>α-Syn/presynaptic maintenance?</td>
</tr>
<tr>
<td>PARK5/4p14</td>
<td>autosomal dominant</td>
<td>UCHL1</td>
<td>UCHL1/polyubiquitin hydrolase</td>
</tr>
<tr>
<td>PARK6/1p36</td>
<td>autosomal recessive</td>
<td>PINK1</td>
<td>PTEN-induced putative kinase-1/mitochondrial protein kinase</td>
</tr>
<tr>
<td>PARK7/1p36.33-36-12</td>
<td>autosomal recessive</td>
<td>DJ-1</td>
<td>DJ-1/mitochondrial antioxidiant, chaperone</td>
</tr>
<tr>
<td>PARK8/12q12</td>
<td>autosomal dominant</td>
<td>LR2K2</td>
<td>Lardarin/multifunctional kinase/GTPase</td>
</tr>
<tr>
<td>PARK9/1p36</td>
<td>autosomal recessive</td>
<td>ATP13A2</td>
<td>Lysosomal type 5 P-ATPase</td>
</tr>
<tr>
<td>PARK10/1p32</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>PARK11/2q36-37</td>
<td>autosomal dominant</td>
<td>GiGYF2</td>
<td>Grb10-interacting GYP protein 2, modulates tyrosine kinase receptor signaling, includs IGF-1</td>
</tr>
<tr>
<td>PARK12/Xq21-q25</td>
<td>X-linked</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>PARK13/2p12</td>
<td>autosomal recessive susceptibility factor</td>
<td>Om/HtrA2</td>
<td>Om/HtrA2, mitochondrial serine peptidase, inhibitor of IAPs</td>
</tr>
<tr>
<td>PARK14/22q13.1</td>
<td>autosomal recessive</td>
<td>PLA2G6</td>
<td>Phospholipase A2 group VI</td>
</tr>
<tr>
<td>PARK15/22q12-ql3</td>
<td>autosomal recessive</td>
<td>FBXO7</td>
<td>F-box protein 7</td>
</tr>
</tbody>
</table>

*From Martin, 2010*

### 3.2.5.1. MONOGENIC FORMS WITH TOXIC GAIN OF FUNCTION

Among all PD-related genes, for SNCA gene (Park1) and LRRK2 gene (Park8), a toxic gain of function has been demonstrated.

**SNCA gene** – the first to be discovered - is located on long arm of chromosome 4, (Park1; 4: q21-q23) and codifies for α-synuclein (α-Syn). Park1 has autosomal dominant transmission, with penetrance lower than 33% (Polymeropoulos et al, 1997); three specific missense point mutation (A53T, A30P and E46K) as well as dominant allele duplications or triplication have been found to cause PD (Singleton et al, 2003).
α-Syn is a small (140 amino acids, 18-20 kDa) very abundant protein (~1% of total protein), found in cells throughout the nervous system and particularly enriched in neuronal axon terminals, where according to growing evidence, it has a role in neurotransmitter release. α-Syn presence in neocortex, hippocampus and substantia nigra seems to be related also to regulation of mitochondrial activity, apoptosis, synaptic plasticity and neuronal differentiation (Kim et al, 2004).

It is a soluble monomeric protein that can associate with mitochondrial membranes and can be induced to polymerize into insoluble fibrils due to a conformational change from an α-helical coil to a β-pleated sheet (Serpel et al, 2000). In most neurodegenerative diseases, LBs are associated with accumulation of wild-type, not mutant, α-Syn.

While recent studies demonstrated how nanomolar concentrations of human wild-type of α-Syn can have a protective effect against cellular stress induced by reactive oxygen species (ROS), hypoxia or glutamate (Seo JH, 2002), over-expression of mutant α-Syn in cultured cells causes mitochondrial deficits, and increases intracellular ROS production, expression of pro-apoptotic proteins and caspases activity (Saha et al, 2000).

Kim et al. also demonstrated that elevated extracellular concentrations of α-Syn induce important release of potent reactive nitrogen species from surrounding microglia, with consequent toxic effect on dopaminergic neurons (Kim et al, 2004).

Mutations of LRRK2 gene on chromosome 12 (Park8; 12: p11-q13), are to date the most common in both familial and sporadic PD (Funayama M et al, 2005). Park 8 codifies for leucine-rich repeat kinase 2 (LRRK2), a large multidomain protein (2527 amino acids, 286 kDa), also called dardarin that is expressed throughout the body.

In nigro-striatal networks, high mRNA levels of dardarin are present in medium spiny neurons, cholinergic interneurons and subpopulations of gabaergic ones, but not in pars compacta dopaminergic neurons (Farrer M. et al, 2005).

Dardarin protein has a predominant mitochondrial localization; it consists of a GTP-ase domain, meaning its possible involvement in cytoskeleton organization and vesicle trafficking, a C-ending domain and a functional kinase domain (Gluo L. et al, 2007). This protein also contains a number of repeated sequences (33-34 aminoacids rich of leucine) at the N-ending that probably mediate protein-to-protein interactions (Figure 6).
Currently it is not evident how LRRK2 gene mutations relate to the selective death of neurons that causes PD. They seem to promote intracytoplasmic inclusions formation, even if the most common missense mutation (G2019S) is usually associated with PCD through apoptotic mechanisms (Iaccarino et al, 2007).

G2019S mutation has high prevalence in Italian population (5% of familial forms, 2% of sporadic ones), thus being the most frequent known cause of PD, and in general the most frequent known cause of neurodegenerative disease (Bonifati, 2006).

3.2.5.2. MONOGENIC FORMS WITH LOSS OF FUNCTION

PARKIN gene (PARK2) was discovered in 1998 (Yamamura et al). It is located on long arm of chromosome 6 (Park2; 6: q25.2-q27) and encodes for Parkin, a ubiquitin E3 ligase.

PARK2 causes 20% of juvenile-onset (before 40 years of age) recessive PD (Kitada et al, 1998) and until 70% of sporadic cases with onset before 20 years of age.

It is characterized by a relatively confined neuronal loss in the SNC and locus coeruleus, but with an absence of LBs (Farrer et al, 2001).

As ubiquitin E3 ligase, this protein mediates covalent bonds between activated ubiquitin and target proteins, destined to proteolytic degradation.

The entire process of ubiquitination - comprising also ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) - will not be described in the text, but is clearly summarized in the figure below (Figure 7).

Even if parkin role of E3 ligase induced to speculate its involvement in proteasomic degradation damage, recent studies revealed its role in intracellular signaling, post-transcription
regulation and protein trafficking (Sriram SR et al, 2005), and in maintaining mitochondrial function (Exner et al, 2007) through protection against oxidative stress (Rothfuss et al, 2009).

Interestingly, abundant presence of ubiquitinated proteins in LBs suggests a possible functional association between parkin and α-Syn (Schlossmacher et al 2002).

![Ubiquitination process and ubiquitine proteasome-system.](image)

Figure 7: Ubiquitination process and ubiquitine proteasome-system.

Another gene called DJ1, located on short arm of chromosome 1 (Park7; 1: p36), has been found to be responsible for 2% of familial recessive PD. Encoded protein has a role in D2 receptor mediated signaling, a chaperone-like activity and is probably a sensor for oxidative stress (Canet-Aviles, 2004).

Neuroimaging and clinical features of genetic PD related to parkin, Pink1 and DJ1 mutations are quite similar, characterized by young onset, slow disease progression and good L-Dopa response. Even if each gene role in neuroprotection is not completely known, they are probably all involved in detection of oxidoreductive cellular homeostasis and mitochondrial function regulation (Klein et al, 2007).
Finally, a number of genetic PD patients are carriers of a single mutation in heterozigosis of Parkin, Pink1 or DJ1. A possible explanation for that is haploinsufficiency mechanism, causing a dominant negative effect of mutated allele.

In conclusion, these heterozygous mutations are an interesting field for further studies on preclinical stages of the disease, underlying molecular pathogenetic and compensatory mechanisms, and consequently for sensitive biomarkers development.

3.2.5.3. GENOMIC ASPECTS OF PD

In parallel with studies on monogenic forms of PD, growing interest developed towards genomic research in patients with sporadic form of the disease.

Emerging data seem to point to a convergence of molecular pathways responsible of dopaminergic neurodegeneration, in particular those regarding mitochondrial dysfunction, altered response to oxidative stress, protein misfolding and compromised proteasomal/lisosomal systems (Figure 8).

![Figure 8 Genes and principal pathways causing genetic PD](image-url)

Figure 8 Genes and principal pathways causing genetic PD
Studies on gene expression profiling in patients with idiopathic PD could potentially contribute to the discovery of susceptibility markers of disease, to be used for preclinical diagnosis, phenotypical characterization and pharmacological responsiveness analysis.

Finally, as preclinical PD phase is estimated to last 5-9 years, there's wide margin of action for potential neuroprotective strategies.

3.2.6. DIAGNOSIS

*United Kingdom Parkinson’s Disease Society Brain Bank* (UKPDS Brain Bank, 1989) are criteria commonly used for PD diagnosis.

A more recent revision of those criteria (Gelb et al, 1999) underlines the importance of both “cardinal” features, present in 69-100% of patients, and “atypical” features considered as exclusion criteria for a more accurate diagnosis of PD.

**UKPDS Society Brain Bank Diagnostic Criteria for Parkinson’s Disease:**

**Step 1: Diagnosis of Parkinsonism**

Bradykinesia and at least one of the following:

- Muscular rigidity
- 4–6 Hz resting tremor
- Postural instability not caused by primary visual, vestibular, cerebellar or proprioceptive dysfunction

**Step 2: Features tending to exclude PD as the cause of Parkinsonism**

- History of repeated strokes with stepwise progression of parkinsonian features
- History of repeated head injury
- History of definite encephalitis
- Neuroleptic treatment at onset of symptoms
- >1 affected relatives
- Sustained remission
- Strictly unilateral features after 3 years
- Supranuclear gaze palsy
- Cerebellar signs
• Early severe autonomic involvement
• Early severe dementia with disturbances of memory, language and praxis
• Babinski’s sign
• Presence of a cerebral tumour or communicating hydrocephalus on computed tomography scan
• Negative response to large doses of levodopa (if malabsorption excluded)
• 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure

Step 3: Features that support a diagnosis of PD (three or more required for diagnosis of definite PD)
• Unilateral onset
• Rest tremor present
• Progressive disorder
• Persistent asymmetry affecting the side of onset most
• Excellent (70–100%) response to levodopa
• Severe levodopa-induced chorea
• Levodopa response for >5 years
• Clinical course of >10 years

Criteria of diagnosis of PD (Gelb et al, 1999) commissioned and supported by the Advisory Council of the National Institute of Neurological Disorders and Stroke, US National Institutes of Health.

Grouping of clinical features of Parkinson’s disease according to diagnostic utility:

GROUP A: Features characteristic of Parkinson disease
• Resting tremor
• Bradykinesia
• Rigidity
• Asymmetric onset

GROUP B: Features suggestive of alternative diagnoses
• Features unusual early in the clinical course
• Prominent postural instability in the first 3 years after symptom onset
• Freezing phenomena in the first 3 years
• Hallucinations unrelated to medications in the first 3 years
Dementia preceding motor symptoms or in the first year
• Supranuclear gaze palsy (other than restriction of upward gaze) or slowing of vertical saccades
• Severe, symptomatic dysautonomia unrelated to medications
• Documentation of a condition known to produce Parkinsonism and plausibly connected to the patient’s symptoms (such as suitably located focal brain lesions or neuroleptic use within the past 6 months)

Criteria for POSSIBLE diagnosis of Parkinson’s disease

At least 2 of the 4 features in Group A are present; at least 1 of these is tremor or bradykinesia
And either:
• none of the features in Group B is present
• or symptoms have been present for <3 years, and none of the features in Group B is present to date
And either:
• substantial and sustained response to levodopa or a dopamine agonist has been documented
• or patient has not had an adequate trial of levodopa or dopamine agonist

Criteria for PROBABLE diagnosis of Parkinson’s disease

At least 3 of the 4 features in Group A are present
And none of the features in Group B is present (note: symptom duration of at least 3 years is needed to meet this requirement)
And substantial and sustained response to levodopa or a dopamine agonist has been documented

Criteria for DEFINITE diagnosis of Parkinson’s disease

All criteria for POSSIBLE Parkinson disease are met
And histopathological confirmation of the diagnosis is obtained at autopsy
Proposed criteria for histopathological confirmation of Parkinson disease

- Substantial nerve cell depletion with accompanying gliosis in the substantia nigra
- At least 1 Lewy Body in the substantia nigra or in the locus coeruleus (note: it may be necessary to examine up to 4 non-overlapping sections in each of these areas before concluding that Lewy bodies are absent)
- No pathological evidence for other diseases that produce Parkinsonism (eg progressive supranuclear palsy, multiple system atrophy, cortical–basal ganglionic degeneration)
  (Note: in excluding other diseases that produce Parkinsonism, published consensus criteria should be used when available)

Even if these standardized criteria reduce diagnostic error at less than 10%, absolute diagnostic accuracy is not guaranteed (Huges et al, 2002). In this scenario, a specific biological marker could significantly increase this parameter.

Other than that, considering the long lasting preclinical phase (5-9 years), the same biomarker could be a precious tool for early diagnosis and potentially for a neuroprotective treatment, capable of modifying natural history of the disease.

Finally, considering phenotypical heterogeneity of PD and frequent overlapping of clinical features with other forms of parkinsonism, a biomarker would significantly help in differential diagnosis.
4. PROJECT n. 1:
GENETIC PARK PROJECT FRIULI-VENEZIA GIULIA

4.1. MULTIFACTORIAL DISEASES

Multifactorial diseases are the result of environmental influence (smoke, nutrition, toxic exposure, etc) upon genetic predisposition. They are also called polygenic, because different independent susceptibility genes contribute to the predisposition of developing the disease.

Hypertension, diabetes, osteoporosis and neurodegenerative diseases like AD and PD are among multifactorial diseases.

There is growing interest on the genetic of these diseases, as the can open new insight into underlying pathological mechanisms, needed to be known for developing preventive and treating therapies.

Isolated populations represent a very useful model to study multifactorial diseases.

4.1.1. ISOLATED POPULATIONS

Isolated populations are those derived from a small number of founders, developed for several generations in a condition of geographical and cultural isolation, without any relevant genetic influence from the outside. Therefore, to be defined as “isolated” a population needs some characteristics, such as:

1. isolated geographical localization
2. few founders and surnames
3. high endogamy rate
4. low immigration/emigration rate

Isolation can happen for several reasons, but the geographical barriers, like mountains and rivers, are the most important ones, as they tend to reduce population mobility and generate ideal conditions for isolation.

Other contributing causes could be unexpected and sudden environmental changes as well as wars and famines, or religious and cultural factors.

Isolation promotes homogeneity of genetic pool: if in a normal population coupling happens casually, in an isolated population it happens between individuals with familiar linkage. In this way, an inbred population is created; the deriving reduced allelic variability increases homozygosis rate which contributes to greater possibility of recessive traits to be clinically expressed.
This is the reason why isolated populations are really useful to study both monogenic and complex diseases (Kristiansson et al, 2008).

Thanks to genetic homogeneity mostly due to endogamy and environmental uniformity, isolated populations represent the ideal condition to identify new genes and study gene/environment interaction in complex diseases (Peltonen et al, 2000; Varilo et al, 2004).

The efficacy of this model has been shown with studies made in Finland, Spain and Island, where several genes responsible for multifactorial diseases have been mapped (Wooley et al, 2002; Varilo et al, 2004; Paunio et al, 2001; Helgason et al, 2004).

The use of isolated populations to reduce heterogeneity of complex and/or quantitative traits has already been proven as useful method to identify DNA polymorphisms associated with these traits (Varilo et al, 2004).

Summarizing, isolated communities, characterized by hundreds of years of isolation and by the maintenance of a traditional life style, make easier not only the recognition of environmental variability influence of phenotypes, but also studying the homogeneous genetic background; inbreeding, typical of small communities, reduces genetic heterogeneity and increases homozygosis, providing greater power for detection of susceptibility genes (Varilo et al, 2004).

As a matter of facts, general reduction in genetic and environmental variation as well as the availability of confined, well-documented extended pedigrees, results in an increase in statistical power to identify genes, particularly in terms of the ability of mapping genetic disease loci using linkage disequilibrium analysis.

4.1.2. MARKERS OF COMPLEX DISEASES

Several genetic variants are used as susceptibility markers:

- Short tandem-repeat (STR) occur when a pattern of two or more nucleotides are repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 50 base pairs (bp) and is typically in the non-coding intron region (Figure 5). A short tandem repeat polymorphism (STRP) occurs when homologous STR loci differ in the number of repeats between individuals. By identifying repeats of a specific sequence at specific locations in the genome, it is possible to create a genetic profile of an individual. They are particularly useful in the study of monogenic diseases, but due to their low density, can be located not close to the genes of interest, and therefore be useless for correlations with specific diseases.

- Insertions and deletions: represent respectively the acquisition or loss of one or more nucleotides in DNA sequence; these are the less frequent genetic variations and commonly occur in non-coding intron regions.
- Rearrangements: are location variations of small portions of a DNA sequence inside a chromosome; they have been used as genetic markers in human genome mapping.

- SNPs (Single Nucleotide Polymorphisms): are the markers most frequently used for genetic analysis. They represent variations of a single nucleotide inside DNA sequence (Figure 9).

![Figure 9: SNPs e STR(P)](image)

SNPs represent 90% of all human genetic variations; they are around 10 million and estimated to be one every thousand of nucleotides couples. Thanks to their high frequency, stability and easy way of detection, SNPs represent a rich source of genetic markers.

SNPs can occur inside a codifying, intronic or intergenic DNA sequence. Therefore, they do not necessarily modify the codified aminoacidic sequence, but can alter gene expression through a change in splicing mechanism or interference with binding to transcription factors.

They represent an important instrument for identification of genes associated with complex diseases, as even small variations in single nucleotides can influence individual responses to pathogens, chemical substances, drugs, but also disease clinical expression.

Even if some SNPs can have a direct impact on the disease, they are often simply a marker for biological diversity, on linkage disequilibrium with the gene really responsible for that given phenotype. SNPs are therefore used in association studies, in order to identify the genetic basis of common diseases with complex aetiology (Ji et al, 2002).

4.1.3. STUDY APPROACHES TO COMPLEX DISEASES

There are different methods for mapping susceptibility genes in multifactorial diseases, depending on sample number, phenotype and known aspects about the tract of interest, like genealogical tree, known candidate genes, etc.

- parametric linkage analysis
- non-parametric linkage analysis
- Linkage Disequilibrium (LD) association studies
**Parametric linkage** analysis is a segregation analysis studying large families with important recurrence of the given disease, in order to assign a gene to a particular chromosomic region, defined by a group of polymorphic markers. If a marker is located close to the gene responsible of the disease, it is supposed that all the members of that family have the same marker allele in linkage with the one responsible of the pathological phenotype. Linkage therefore means that the disease-related gene maps close to the discovered marker. In order to define the existence of a linkage, several families with many affected members each are needed, as well as a specific hereditability model.

For all these reasons, linkage analysis is very useful to identify genes responsible for monogenic diseases, but is not an efficient method to discover genes related to complex diseases.

**Non-parametric linkage** methods are based on the study of Identical-By-Descent (IBD-sharing or allele-sharing) shared DNA regions, in order to identify susceptibility genes in affected individuals. In this approach, the analysis is focused only on the IBD transmission of genetic markers inside families. If a given chromosome region contains a susceptibility gene for a tract of interest, all affected members of the family will share the IBD with greater probability than what expected with an independent transmission. That difference can be calculated with statistical methods used in IBD-sharing analysis.

**Association studies (or Linkage Disequilibrium-LD)** are based on a statistical measure that shows the non-causal association between alleles in loci located not far to each other in the chromosome. They compare the allele frequency in a sample of unrelated affected subjects with that in a sample of healthy controls. They do not compare individuals belonging to the same family, and do not require a priori information, such as the hereditability model of the tract object of the study. The basic hypothesis of an association study is that the presence of genetic polymorphisms (SNPs) is related to increased risk of developing complex diseases. Obviously, there is an association when a specific allele is more frequent in affected than control subjects.

Mapping through association is a very efficient approach in the study of complex diseases in small and homogeneous populations (Cardon et al, 2001).

A Genome–wide association study (GWAS) is a study made on the entire genome, in order to analyse its genetic variations. It consists of evaluation of the genome in different individuals, obtained using the highest possible number of genetic markers without any a priori gene knowledge and/or selection (Hirschhorn et al, 2005).

A GWAS implies the choice of cases and controls, the selection of SNPs spread along all the genome, and their genotypization. Its objective is identifying genetic associations with observable traits or the presence/absence of a disease/condition.
In addition, rapid advances in understanding the patterns of human genetic variation and maturing high-throughput, cost-effective methods for genotyping are providing powerful research tools for identifying genetic variants that contribute to health and disease.

4.1.4. GENOTYPIZATION

SNPs genotypization demonstrated to be the most efficient approach to susceptibility genes identification inside isolated populations (Ji et al, 2002). As SNPs are markers to localize a gene inside a DNA sequence, those present in an affected population can be compared with those of a healthy one. The specific SNP more represented in the case population can be used disease-related gene localization.

Linkage disequilibrium means the tendency of specific alleles, in two or more closely associated loci, to be localized in the same chromosomal tract, being in this way transmitted together along the genealogic tree. This group of alleles is called haplotype; haplotypes are recognizable chromosomes segments that can be followed along genealogic trees and populations.

In isolated populations, segments of ancestral chromosomes are recombined during many generations; those segments that are not, can be found in several members of the same population and can be useful for identifying disease-related genes. In isolated population, due to inbreed, affected subjects usually descend from the same ancestor carrier: despite many generations and meiosis, that mutation inside that haplotype usually remains the same.

In the study of complex diseases, linkage disequilibrium is applied in affected subjects in order to identify with genetic markers located close to the disease-related gene, those ancestral haplotypes (Weiss et al, 2002).

There are up to 70 SNPs inside a single haplotype that are not subject to recombination, remaining therefore close and being inherited together.

For each haplotype is thus possible to select a limited number of SNPs, the so called tagSNPs: in this way the number of SNPs to be used in genotyping is reduced from 10 millions to 1 million-200 thousands tagSNPs.

TagSNPs are available thanks to the HapMap; the International HapMap Project (Nature, 2003) was designed to create a public, genome-wide database of patterns of common human sequence variation to guide genetic studies of human health and disease. With the publication of the draft human genome sequence in 2001 and the essentially finished version in 2003, the HapMap emerged as a logical next step in characterizing human genomic variation, particularly of the millions of common single–base pair differences among individuals (Manolio et al, 2008).
Genotypization with SNPs allows defining those haplotypes that in a given population segregate with the disease, thus defining a region carrier of a susceptibility gene. Inside this region, each single gene considered relevant to the disease need thereafter to be sequenced, in order to establish if there is a real relationship with the disease. If this happens, that specific gene has to be found in each affected subject.

We have decided to combine the power of a Genome Wide Association Study (GWAS) with the potential of isolated populations to study dementia and possible underlying genes in six isolated populations from North Eastern Italy (Friuli Venezia Giulia).

4.2. DESCRIPTION OF THE STUDY

4.2.1. FRIULI VENEZIA GIULIA GENETIC PARK PROJECT

Friuli Venezia Giulia Genetic Park is a project born in 2008, whose aim is to realizing a regional Biobank with clinical, historical, environmental and biological data, for identification of genetic basis of complex diseases such as hypertension, obesity, deafness, osteoporosis, as well as psychiatric and neurodegenerative diseases. This project is part of the Italian network of isolated Populations, which involves several research institutes in Alto Adige, Piemonte, Veneto, Lombardia, Campania and Puglia.

Our purpose was to study these diseases among the population of six towns that have been isolated for a long time, due to geographical, historical, linguistic and/or cultural reasons. They are located in the province of Udine (Illegio, Sauris, Resia), Pordenone (Clauzetto, Erto-Casso) and Gorizia (San Martino del Carso) (Figure 10).
Figure 10: Map of Friuli Venezia Giulia, with towns involved in the project

These towns have been identified as candidates for this study thanks to their isolation and consequently uniform genetic background; they are in fact characterized by isolated geographical localization, small number of founders, few surnames, high endogamy rate and low emigration/immigration rate, sometimes by a linguistic barrier.

Local administration, general practitioners and religious authorities gave approval to this project; all inhabitants were invited, and participation was voluntary.

4.2.2. PURPOSE OF THE PROJECT

All dementias, but AD in particular, have progressively become a more and more important issue for public health.

Dementia prevalence in Italy is around 5% in subjects over 65 years of age, reaching 40% in subjects older than 85 (Ravaglia et al, 2008).

The progressive increase in elderly population obviously favors a growing incidence.

Dementia means degeneration, which not only involves cognition, personality, relationships and global functioning of the subjects, but also generates huge economical and human costs to the society, related to quality of life and mortality of the patients, as well as psychophysical health of the caregiver. A subject with dementia requires not only an accurate diagnostic and therapeutical approach, but also an adequate assistance throughout the course of the disease, stimulating when possible the residual resources of the individual and taking care of all his needs, especially in the last phases of the disease (Jönsson et al, 2006).
The psychological, emotional, social and economical impact of the disease on the family is a heavy burden that compromises habits, social relationships and consequently the quality of life (Papastavrou et al, 2007).

Caregivers themselves are therefore at risk for developing physical or mental illness, and there is among them a greater incidence of depression and alcohol abuse among them (Polen et al, 2001).

Finally, economical and social costs are dramatic also for the entire community: it has been calculated that each demented patient in the initial phase of the disease costs around 40 thousands Euros to the society, increasing to 57 thousands in the last phase (Gambina et al, 2003).

Genetic research can therefore contribute, through identification of genes involved, to a better understanding molecular pathways involved in pathogenesis, possibly leading to the discovery of new preventing/protecting/healing therapy for neurodegeneration. So far only symptomatic drugs exist, which can only temporary ameliorate cognitive performances, but do not arrest degeneration precess.

For all these reasons, the Friuli Venezia Giulia Genetic Park Project had several purposes:
1. Find out dementia prevalence in these populations
2. Estimate the prevalence of some dementia risk factors
3. Evaluate the specific contribution of both genetic and environmental factors in Alzheimer's Disease.

All clinical and genetic data collected, non only by neurologist but also from other involved specialists, represents a sort of genetic mapping of the examined pathological conditions, and could therefore be used in the future as a starting point toward correlation analysis between genotype, phenotype and style of life.

4.3. MATERIALS AND METHODS

4.3.1. TOTAL SAMPLE

Sample enrolled in the study belongs to six different isolated populations in North-Eastern Italy (Friuli Venezia-Giulia):

a) Sauris (423 inhabitants), whose language is ancient German;

b) Resia Valley (1175 inhabitants) whose language is an archaic Slavic dialect of unknown origin;
c) Illegio (410 inhabitants) in Carnia region;
d) Clauzetto (404 inhabitants);
e) Erto-Casso (338 inhabitants) lasting from the dramatic collapse of the Vajont Dam;
f) San Martino del Carso (232 inhabitants) in which one family name accounts for the great majority of living people, who speak a Venetian dialect; Slovenian communities surround this village.

Meetings with authorities and population were organized in each town, to explain the different phases of the study; brochures with detailed descriptions of it have been distributed to the entire population.

A total of 1137 participants were randomly chosen. The research project respected all the Italian laws and rules in terms of privacy, biological samples collection and storage, and DNA test. Project and the protocols were approved by the Ethical Committee of IRCCS-Burlo Garofolo.

During the first medical visit, each subject received detailed information about methods and purposes of the study, and signed an informed consent.

General project-wise, anthropological measures (height, weight), clinical data (e.g. blood pressure, past medical history), and information about lifestyle (e.g. job, toxic exposure, habits, etc) were gathered.

Each subject was invited to cardiological, psychiatric, nutritional, stomatological, audiometrical and neurological evaluation.

All clinical and genealogical data where thereafter collected in a proper database and a code number has been assigned to each subject for identification.

Exclusion criteria for neurological analysis were: a) age below 40 years b) lack of information about education c) lack of neurological examination d) lack of neuropsychological screening.

Neurology wise, our goal was to find out all individual possibly affected by dementia, in order to compare each town’s prevalence with that of general population. Five neurologists, used to evaluation of patients with dementia and parkisonisms, participated as examiners to this part of the study, which was subdivided in:

1. Data collection about the subject’s personal and medical history, with greater attention on possible cardiovascular risk factors, as well as familiar/personal history of cognitive problems;
2. Clinical assessment to evaluate motor and cognitive status, through a short neurological examination (mostly focused on pyramidal and extrapyramidal signs) and neuropsychological screening tests; as we will be described later in the text, these data has been used to identify all demented subjects, and among them, those possibly affected by AD and mixed dementia;

As dementia was our dependent variable, we chose as covariates sex, age, education, and village. Education was divided in four categories: elementary, primary school, secondary school, and graduation.

The effect of these covariates on dementia was tested by linear regression for continuous variables or logistic regression for dichotomous ones.

4.3.2. "NEUROLOGICAL" SAMPLE

A total of 920 individuals therefore were evaluated by neurologists.

As previously described, each subject underwent familiar, personal, past medical history, as well as history of present illness, considering in the latest the presence of cognitive decline. All data about familial/personal history of neurological diseases (dementia and movement disorders in particular), cardiovascular risk personal profile, nutrition, habits, toxic exposure, medications, job, and head traumas were collected.

Subjects then underwent a short neuropsychological screening; for this purpose we decided to use Mini Mental State Examination (Folstein et al, 1975) and Ten Point Clock Test (Manos et al, 1994).

Mini Mental State Examination (MMSE) is a very common screening test in clinical settings, useful and reliable, especially for first contact with a patient with mental deterioration. However, it has several weaknesses: it lacks sensitivity for the early stages of dementia, as presents “ceiling effect” resulting in some false-negative diagnosis in mild AD, but also has a “floor effect” for advanced stages of AD for the difficulty to assess memory, language and perceptual problems in severely impaired patients; it’s not a reliable test for patients with prominent language problems, because it is mostly based on verbal tasks, so can overestimate the cognitive deficits; there’s only a short general assessment of memory – with no cueing and recognition paradigms, no visual, personal and working memory measures; only superficially evaluates attention; does not have any task to assess executive functions, like abstract reasoning or judging social situations, that are both early compromised in AD; can cause interpretation problems, due to practice effect with repetitive testing, especially in mild AD.

Although it has all these weaknesses, MMSE still remains a: it rapidly provides data that illustrate severity of cognitive decline and is a universally known instrument providing information easy to communicate and to be interpreted for professionals. It is also useful to monitor patient’s decline and/or efficacy of a given therapy (Mitchell et al, 2009).

Through simple questions and tasks, it allows exploring the following cognitive domains: temporal and spatial orientation, immediate and short delayed recall, attention and calculation,
language (written and oral commands comprehension/execution, naming, reading, writing), visuospatial functions.

MMSE score (if < 24/30) has a sensibility of 80-90% and a specificity of 80% in distinguishing people with and without cognitive decline (Fountoulakis et al, 2000); a score between 25 and 30 means absence of cognitive decline, from 19 to 24 mild dementia, from 11 to 18 moderate dementia, severe dementia if below 10 (Ward et al, 2002).

As age and education contribute significantly to MMSE variations expected in normal populations, we applied validated correction factors to the row scores (Magni et al, 1996).

MMSE scores of subjects with AD usually progressively decline of 1.8-4.2 points every year (Clark et al, 1999; McCarten et al, 2004).

A study conducted by Ashford et al in 1989, studying 86 patients meeting DSM-III criteria for primary degenerative dementia (average age 74±8 and average education 11±30), and using logistic regression analysis, described the progressive loss of some items of MMSE. In the mild stage of dementia, items with the highest severity of impairment are those related to recent memory and attention: recall of three objects, recall of the date and serial seven calculations. In moderate dementia, memory and orientation for time and place are impaired. In the severe stage of the disease, lost items include repetition of words, naming simple objects and following commands are compromised. Naming objects, writing and reading are usually lost later in AD than repetition and command items. For both early and long standing AD, items “recall” and “copy of figure” are the most difficult to be performed successfully.

TPCT is a useful neuropsychological screening tool like MMSE, and with a good correlation with it (Manos et al, 1994). It is very rapid and easy to be administered, and instructions not difficult to be understood. He mostly evaluates visuospatial and executive functions, abstract reasoning and symbolic representation. There are two ways to propose this test: the “free-drawn” method asks the patient to design a clock recalled form his memory, while in the “pre-drawn” method (used in this study) a paper with a circle already drawn is presented to the patient; he is supposed to draw correctly numbers of hours and after that minute and hour hands, pointing at 11.10 or 2.45. (Peters et al, 2008). Quantitative and qualitative evaluation is made on number position, on ability of planning and organizing space, and on abstract reasoning used to place hands with the proper length and direction. Score ranges from 0 to 10, and cognitive decline defined as unlikely (score 10), probable (score 8 or 9), definite (score<8) and prominent (score<5).

TPCT can correctly classify 77% of AD patients and 89% of patients with vascular dementia; the latest in particular usually make more mistakes on organizing space (Agrell et al, 1998). Like MMSE, this test does not have enough sensitivity to identify very early cognitive decline, and is influence by age, education and language (Pinto et al, 2009).
The last part of the neurological evaluation was a brief neurological examination, with particular attention on pyramidal and extrapyramidal signs, as they can help in the differential between AD and other forms of dementia (e.g. vascular dementia, Parkinson Plus syndromes, etc).

4.3.3. DNA SAMPLE

Venous blood samples were collected from 1737 inhabitants of the six villages, including those born in FVG but living abroad.

Basic hematological parameters, such as leucocytes and erythrocytes count, hemoglobin, hematocrit, mean corpuscular volume, platelets glucose, lipid profile, renal and liver function, electrolytes where analyzed.

DNA of 1376 samples was extracted from peripheral blood by automated purification methods using EZ1 workstation of Quiagen. DNA quantity and quality of DNA were misread using NanoDrop. Sera and RNA were also collected in order to perform additional analysis. All samples were genotyped with Illumina 370k platform and then imputed using MACH to a common set of ~2.5 million autosomal SNPs based on LD patterns observed in Hap Map CEU reference samples. Imputed allele dosage and genotyped SNPs were used to analyze the quantitative genetic trait.

4.3.4. STATISTICAL ANALYSIS

Because of the presence of close relatives in our dataset, statistical analyses were performed using a mixed model regression as implemented in GenABEL and ProbABEL. Kinship was estimated through as implemented in GenABEL. This package is a library of R, very useful for managing a great amount of data. GeneABEL provides all instruments for more common and important statistical analyses, allows to realize graphics and tables, and to calculate SNPs frequency in a population, finding out associations between a given tract and a genetic polymorphism. It is therefore very useful on selecting genetic markers of interests in GWAS (Aulchenko et al, 2007).

Through this software, genotype frequencies in each population and possible associations between high frequency SNPs and dementia were analyzed.

As statistical test we used Family-based Score Test for Association (FASTA) (Chen et al, 2007) and genetic model was presumed to be additive using sex, age, education and village as covariates. We included only SNPs with a 90% genotyping rate, with Minor Allele Frequency (MAF) ≥0.05 and Rsq>0.3.
4.4. RESULTS

4.4.1. EPIDEMIOLOGICAL ANALYSIS

A total of 920 subjects older than 40 years of age were evaluated; male/female rate was 385/535, mean age 59.7 ± 12.2 years, range 40-91 years; 612 subjects (66.5%) were ≤65 years, 308 (33.5%) were >65 years.

Considering each town, 280 individuals lived in Resia, 149 individuals in Clauzetto, 142 in San Martino del Carso, 121 in Erto and Casso, 119 in Illegio, 109 in Sauris (Table 1).

<table>
<thead>
<tr>
<th>Town</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resia</td>
<td>280</td>
</tr>
<tr>
<td>Clauzetto</td>
<td>149</td>
</tr>
<tr>
<td>Erto e Casso</td>
<td>121</td>
</tr>
<tr>
<td>Illegio</td>
<td>119</td>
</tr>
<tr>
<td>Sauris</td>
<td>109</td>
</tr>
<tr>
<td>San Martino del Carso</td>
<td>142</td>
</tr>
</tbody>
</table>

Table 1: Epidemiological characteristics and distribution of evaluated subjects

89 subjects (9.7% of population examined) satisfied diagnostic criteria of possible dementia, 2.4% of those ≤65 years (n=15) and 24% of those >65 years (n=74). Among individuals older than 80 years (n=42), 54.5% (n=23) showed a cognitive decline.

Considering scores on TPCT, 89% (n=546) of subjects ≤65 years and 65% (n=199) older than 65 years had a normal score, while a pathological one was obtained by 10% (n=61) of those ≤65 years and 32% (n=99) of those >65 years.

Prevalence of possible dementia in each of the six towns examined had the following distribution: Erto-Casso 14.8% (n=18), Sauris 12.8% (n=14), San Martino del Carso 12.6% (n=18), Clauzetto 9.4% (n=14), Resia 7.1% (n=20), Illegio 4.2% (n=5) (Table 2; Figure 11).
Table 2: Distribution and prevalence of subjects with dementia in each community

<table>
<thead>
<tr>
<th>Town</th>
<th>Tot affected</th>
<th>≤ 65 years</th>
<th>&gt; 65 years</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erto-Casso</td>
<td>18</td>
<td>6</td>
<td>12</td>
<td>14.8%</td>
</tr>
<tr>
<td>Sauris</td>
<td>14</td>
<td>1</td>
<td>13</td>
<td>12.8%</td>
</tr>
<tr>
<td>S Martino</td>
<td>18</td>
<td>5</td>
<td>13</td>
<td>12.6%</td>
</tr>
<tr>
<td>Clauzetto</td>
<td>14</td>
<td>1</td>
<td>13</td>
<td>9.4%</td>
</tr>
<tr>
<td>Val Resia</td>
<td>20</td>
<td>2</td>
<td>18</td>
<td>7.1%</td>
</tr>
<tr>
<td>Illegio</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>4.2%</td>
</tr>
</tbody>
</table>

Figure 11: Prevalence of dementia for classes of age in each community

These results seem to point to a higher prevalence of dementia in these isolated populations in comparison to literature data (9% versus 5% of general Italian population) (Ravaglia et al, 2008).

We also wanted to estimate the prevalence of some common cardiovascular risk factors, and analyze their possible association with dementia. We focused our attention on smoke, hypertension, diabetes, dislipidemia, atrial fibrillation and low education (<8 years); due to high consumption of alcohol in these communities, alcohol was added as independent variable to our analysis.
We therefore compared the group of cases (89 subjects with dementia) with controls (831 subjects with no cognitive decline), finding statistically significant differences for some independent variables (Table 3).

Odds Ratio was calculated for each risk factor, in order to estimate a possible association with dementia; according to an Interval confidence of 95%, a positive relationship was found between dementia and hypertension (OR=2.8), atrial fibrillation (OR=5) and low education (OR=8.2).

Even if diabetes has an OR>1 too, its association with dementia is probably casual and not statistically significant, as indicated by IC.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Cases Prevalence</th>
<th>Controls Prevalence</th>
<th>OR</th>
<th>Conf.Int.95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMOKE</td>
<td>3% (n=3)</td>
<td>18% (n=147)</td>
<td>0.2</td>
<td>0.05 &lt; CI &lt; 0.5</td>
</tr>
<tr>
<td>HYPERTENSION</td>
<td>51% (n=46)</td>
<td>28% (n=230)</td>
<td>2.8</td>
<td>1.8 &lt; CI &lt; 4.3</td>
</tr>
<tr>
<td>DIABETES</td>
<td>10% (n=9)</td>
<td>5% (n=42)</td>
<td>2.1</td>
<td>0.9 &lt; CI &lt; 4.5</td>
</tr>
<tr>
<td>DYSLIPIDEMIA</td>
<td>14% (n=13)</td>
<td>11% (n=95)</td>
<td>1.3</td>
<td>0.7 &lt; CI &lt; 2.5</td>
</tr>
<tr>
<td>ATRIAL FIBRILLATION</td>
<td>12% (n=11)</td>
<td>3% (n=23)</td>
<td>5</td>
<td>2.3 &lt; CI &lt; 10.5</td>
</tr>
<tr>
<td>ALCOOL</td>
<td>43% (n=39)</td>
<td>47% (n=390)</td>
<td>0.9</td>
<td>0.5 &lt; CI &lt; 1.3</td>
</tr>
<tr>
<td>EDUCATION &lt;8 ys</td>
<td>95% (n=85)</td>
<td>72% (n=599)</td>
<td>8.2</td>
<td>2.9 &lt; CI &lt; 22.5</td>
</tr>
</tbody>
</table>

Table 3: Risk factors prevalence, Odds Ratios and Interval Confidence

4.4.2. GENETIC ANALYSIS

As told before, the objective of this study was not only evaluating dementia prevalence in isolated populations, but also looking for possible unrevealed genes that can open new horizons on dementia pathogenesis.

Number of dementia subtypes, higher AD prevalence among them, small population examined as well as limited instrumental possibilities due to organizational and logistic matters, guided us to focus on AD phenotype.

For this purpose, we therefore decided to “purify” as much as possible the group of subjects with dementia, by a clinical selection of AD cases realized through accurate analysis of information obtained by interview, clinical assessment and neuropsychological screening.
For AD diagnostic criteria, we referred to NINCDS-ADRDA ones, previously described. The same approach and NINDS-AIREN criteria were used to exclude from our sample individuals with suspicion of vascular cognitive decline.

Subjects were therefore considered affected by this form cognitive impairment, in the presence of the following characteristics:

- History of at least 2 or more among cerebro/cardiovascular events and cardiovascular risk factors: hypertension, diabetes, atrial fibrillation, dislipidemia
- Low score on TPCT ($\leq 5$) but MMSE $\geq 24$, as the former is more sensible to executive dysfunction
- Supportive criteria: bilateral parkinsonism or pyramidal signs on neurological examination

Due to difficulty in some cases of a clear differential between degenerative and vascular dementia, and also to increase number of subjects to be studied, possibly without altering the results, a decision was taken of including in the study group individuals with AD plus cerebrovascular disease (CVD).

Inclusion criteria with possibly greater specificity for AD or AD plus CVD were therefore considered:

- MMSE $\leq 24$ plus TPCT $\geq 7$
- Less than 2 cardiovascular risk factors

According to this empiric selection criteria, we created a sub-population of 49 subjects, potentially affected by AD or AD plus CVD.

From statistical analysis, 8 markers among all SNPs employed obtained a statistically significant association with AD ($p$ value $<10^{-6}$) (Table 4).

They are all located on chromosome 9 (Figures 12 and 13) and have a MAF $>0.1$. MAF means Minor Allele Frequency, which is the lowest allele frequency at a locus observed in a particular population. This is simply the lesser of the two alleles frequencies for single-nucleotide polymorphisms. Values range $0+0.5$ (0.5 means the most significant).
Results can be seen in the Manhattan Plot (Figure 12), where the y-axis represents the different chromosomes, while the x-axis represents log of p-value; that means the higher is the position, the stronger is the significance of genetic association.

Table 4: SNPs statistically relevant and their position on chromosome 9, p-value and MAF

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Chromosome</th>
<th>Location</th>
<th>P-value</th>
<th>MAF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs914804</td>
<td>9</td>
<td>9400073</td>
<td>6.64E-06</td>
<td>0.199116</td>
</tr>
<tr>
<td>rs1556523</td>
<td>9</td>
<td>9402293</td>
<td>5.27E-06</td>
<td>0.197459</td>
</tr>
<tr>
<td>rs1556463</td>
<td>9</td>
<td>9409666</td>
<td>5.48E-06</td>
<td>0.19847</td>
</tr>
<tr>
<td>rs1332207</td>
<td>9</td>
<td>9410712</td>
<td>5.36E-06</td>
<td>0.198434</td>
</tr>
<tr>
<td>rs1332206</td>
<td>9</td>
<td>9410714</td>
<td>5.33E-06</td>
<td>0.198384</td>
</tr>
<tr>
<td>rs1332204</td>
<td>9</td>
<td>9410735</td>
<td>5.30E-06</td>
<td>0.198349</td>
</tr>
<tr>
<td>rs4740981</td>
<td>9</td>
<td>9410015</td>
<td>5.25E-06</td>
<td>0.19832</td>
</tr>
<tr>
<td>rs4742595</td>
<td>9</td>
<td>9410915</td>
<td>5.20E-06</td>
<td>0.198249</td>
</tr>
</tbody>
</table>

Figure 12: Manhattan Plot
After these results, genome around those SNPs found was analyzed, in order to identify possible genes involved in AD susceptibility.

In this way, PTPRD (Protein Tyrosine Phosphatase Receptor Type D) gene located on chromosome 9p23-p24.3 was discovered (Figure 14)
4.5. PTPRD GENE

4.5.1. STRUCTURE AND FUNCTION OF THE PROTEIN

Protein codified by PTPRD gene belongs to phosphatase proteins family; these enzymes remove a phosphate group from its substrate by phosphoric acid monoesters hydrolysisation into a phosphate ion and a molecule with a free hydroxyl group. This action, essential for cyclic activation/deactivation of the targeted protein, is balanced by phosphorylases and kinases, which attach phosphate groups to their substrates by using energetic molecules like ATP. These opposite mechanisms are the basis for signal transduction, allowing cellular growth, differentiation and metabolism.

Protein phosphorylation is the most common and important form of reversible protein post-translational modification (PTM), with up to 30% of all proteins being phosphorylated at any given time. Protein kinases (PKs) are the effectors of phosphorylation that catalyse the transfer of a γ-phosphate from ATP to specific amino acids on proteins, predominantly on Serine, Threonine and Tyrosine residues. In contrast, protein phosphatases (PPs) are the primary effectors of dephosphorylation and can be grouped into three main classes based on sequence, structure and catalytic function. The largest class of PPs is the phosphoprotein phosphatase (PPP) family, comprising also the proteins phosphatase Mg²⁺- or Mn²⁺-dependent (PPM), followed by Tyr phosphatase (PTP) super-family forms the second group, and the aspartate-based protein phosphatases the third (Kellie, 2003).

According to their structure, PTP can be subdivided into two groups: trans-membrane PTP (receptor-like), also called PTPR, and cytoplasmic PTP.

PTPRD protein belongs to the first group and contains an extracellular region with three immunoglobulin-like domains and eight fibronectin type III domains, a single transmembrane domain and two catalytic intracytoplasmic domains (Kellie, 2003).

4.5.2. CLINICAL IMPLICATIONS

PTPRD gene has several clinical implications:

- It is an oncosuppressor, whose inactivation is involved in oncogenesis. PTPRD gene sequencing in different neoplasms revealed its mutation in more than 6% of multiforme glioblastomas, in 13% of squamocellular carcinomas of head and neck and in 9% of pulmonary
carcinomas (Veeriah et al, 2009). Microdeletions of this gene are involved in the pathogenesis of two neoplasms of neuroectodermal origin (melanoma and neuroblastoma) (nair et al, 2008; Solomon et al, 2008).

- PTPRD polymorphisms seems to be related to an increased risk of pediatric asthma (Shyur et al, 2008).
- A GWAS in 2009, focused on genetic determinants for hyperomocysteinemia, did found and association between PRPRD gene and an adjacent SNP (Mälarstig et al, 2009).
- A linkage analysis done in 2008, whose purpose was identifying susceptibility genes for Restless Legs Syndrome, found an association with PTPRD gene (Schormair et al, 2008).
- A recent study (Lei et al, 2010) involving 943 families with almost one child affected by autism, revealed that a particular deletion of PTPRD segregates in the majority of these families, thus suggesting a possible association with the disease.
- Four different deletions of PTPRD have been discovered through linkage genetic studies, focused on ADHD (Attention Deficit Hyperactivity Disorder) susceptibility genes (Elia et al, 2010).

4.5.3. PTPRD AND CENTRAL NERVOUS SYSTEM

In mice, PTPRD gene is involved in formation and maintenance of synapses, by promoting regeneration and axonal transport. It is therefore an important regulator of neuronal plasticity and is implicated in memory and learning processes. It is also highly expressed during formation and growth of CNS in mammals (Uetani et al, 2006).

Studies on mice identified a homologous gene of the human PTPRD, called MPTPD. In situ hybridization on brain samples from mice models, using antibodies against extracellular domain of phosphatase, revealed MPTPD expression in specialized areas such as CA2 and CA3 areas of hippocampus, thalamic reticular nucleus and piriform cortex (Mizuno et al, 1993), olfactory bulb and spinal motor neurons (Sommer et al., 1997; Schaapveld et al., 1998).

In order to gain further insight into physiological role of PTPRD gene in the brain, Uetani et al. in 2002 created PTPRD-deficient mice by gene targeting. Since birth, these animals exhibited growth retardation, severe motor dysfunction, death due to insufficient food intake. Learning and memory abilities evaluated through tests like Morris water maze, reinforced T-maze and radial arms maze tasks, were shown to be impaired in comparison to healthy mice, thus confirming PTPRD involvement in memory and spatial learning (Uetani et al, 2000). Interestingly, the histology of the hippocampus appeared normal.
To assess the mechanism underlying impaired memory and learning, authors performed and electrophysiological analysis of CA1 and CA3 hippocampal areas. In both regions, no significant electrophysiological alteration was observed in PTPRD-deficient mice, implying that neither the number of synapses nor basal synaptic transmission is affected.

Paired-pulse facilitation (PPF, double pulses applied to synaptic input at intervals of 30-950 ms) was therefore performed: PPF amplitude was significantly increased in CA1, but not in CA3 region, suggesting that PTPRD is involved in regulation of synaptic activity in CA1. Given that PTPRD is predominantly expressed at CA2-CA3 pyramidal neurons, the enhanced PPF in CA1 seems to be caused by a pre-synaptic mechanism, which was explained by authors as a consequence of the delayed intracellular Ca\(^{2+}\) decay found in PTPRD-deficient mice (the threshold of the pulse interval causing facilitation was prolonged). This was the first demonstration of a specific PTPRD role in modulating channel activities that control intracellular Ca\(^{2+}\) concentrations.

Authors then examined synaptic plasticity in CA1 (as involved in memory and learning), and found after tetanic stimulation that the magnitudes of long-term potentiation (LTP) induced at CA1 and CA3 synapses were significantly enhanced in PTPD-deficient mice.

Their conclusion was that PTPRD seems to have an inhibitory role in LTP, probably through a pre- and post-synaptic mechanism: (1) because PTPRD is expressed in CA2 and CA3 regions, increased LTP in CA3 on PTPRD-deficient mice is consistent with a post-synaptic effect; (2) PTPRD deficiency may result in facilitation of LTP in CA1, due to increased transmitter release during titanic stimulation, and consequent increased Ca\(^{2+}\) influx at post-synaptic sites, through NMDA and voltage-gated Ca\(^{2+}\) channels with increased paired-pulse facilitation in the CA1 region.

Thus, authors showed that PTPD plays important roles in regulating hippocampal synaptic plasticity.

To explain why impaired learning and memory processes (related to LTP amplitude in CA1) in PTPRD-deficient mice did not correspond to any alteration in hippocampal histology, authors suggested that PTPD deficiency-induced hyperphosphorylation of tyrosine residues of proteins in hippocampal neurons is harmful for the acquisition of a new memory. One possible scenario is that in PTPD-deficient mice, proteins involved in the regulation of ion channel activities of the synapses are hyperphosphorylated in all the neurons, resulting in loss of conductivity difference among synapses and setting the conductivity at elevated levels. This finally leads to a decrease in the synaptic plasticity and contributes negatively to the acquisition of a new memory. Thus, optimal synaptic potentiation needs to be tightly regulated by PTKs and PTPs, and PTPD is one of the key enzymes in this process (Uetani et al, 2000).

A previous study in 2002 aiming at clarifying the role in learning of another phosphatase, called PTP\(\alpha\), find out that the blockade of endogenous PTP\(\alpha\) inhibits NMDA receptor activity and the induction of long-term potentiation in CA1 hippocampal neurons (Lei et al, 2002)
Memory is a complex brain process that allows retaining information. Can be didactically subdivided into long-term and short-term memory.

- **Long-term memory** has a variable duration; lasts from minutes to decades, and comprehends declarative (=explicit) and procedural (=implicit) subtypes. Declarative memory can be moreover subdivided into episodic and semantic memory (Ofen et al, 2007). (a) The former lasts from minutes to years and allows recalling events happened in a particular time and place; the combination of all episodic memories helps a subject to behave also according to previously experiences. (b) Semantic memory refers to general knowledge of concepts acquired during life, not related to a particular personal experience.

  Procedural memory is involved in learning and retaining abilities and/or procedures, such as riding a bike, dressing himself, driving a car, etc. Once learned, these abilities become automatic and they do not need a conscious effort to be recalled. This memory develops slowly, usually through several repetitions, and is expressed through actions, not words (Schacter et al, 1992).

- **Short-term memory** allows to retain only few information (5-9 elements) for a short amount of time (minutes); it is represented by working memory, which is a temporary storage system for information to be then elaborated, deleted or retained. This memory is very vulnerable to distractive factors, that means requirement of alert and attention for it to be maintained (D'Esposito et al, 2007).

Memory in general is the result of different processes:

1. **Encoding**: through attention and motivation, this process analyzes new information as soon as they reach the subjects, establishing role-entity binding; retention of memory trace depends on coding efficiency (pattern recognition machine)

2. **Consolidation and retention**: all those processes that slowly modify learned information in order to stabilize and make them less prone to disruption and forgetting; NMDA receptors in CA1 play a critical role in memory consolidation. During storage of memory traces, hippocampal system to cortex transfer allows episodic memory traces of memorable events persist in the hippocampal system, even after they have contributed to cortical semantic representation.

3. **Retrieval**: recall of information stored; this is possible by activating the web of procedural, semantic and sensory-motor knowledge with the relevant role-entity bindings. An event’s episodic memory trace becomes active and reinstates the event’s bindings within cortical circuits; upon being activated with the appropriate bindings, cortical circuits encoding action
schemas, sensorimotor programs and semantic knowledge reconstruct an event via embodied mental stimulation and reflexive inference.

New information are collected and elaborated at first in associative cortices (posterior parietal, secondary visual, secondary somatosensory, secondary auditory, prefrontal). Then information are transferred to parahippocampal and perirhinal cortices, entorhinal cortex, cingulated gyrus, hippocampus, subiculum and then back to entorhinal cortex. From here, information are transferred again to parahippocampal and perirhinal cortices, and then to neocortex associative areas, which represent the final storage of mnemonic traces.

Hippocampus has therefore a crucial role on memory process, as it is involved in particular on working and declarative memory consolidation. Its disruption blocks new information acquisition, but does not eliminate previously stored memories (Shastri et al, 2002).

Hippocampus is part of limbic system, located inside the medial temporal lobes, and can be subdivided in three distinct areas:

- Dentate gyrus, subdivided in CA3, CA2 and CA1 areas
- Subiculum, the most inferior component of the hippocampal formation, located between entorhinal cortex and CA1 area
- Entorhinal cortex, part of hippocampal cortex and the main interface between the hippocampus and neocortex

Inside hippocampal formation there’s a multisynaptic circuit that from entorhinal cortex goes to granular neurons of dentate nucleus, through fibers called perforant path. Dentate gyrus neurons have axons called muscoid fibers, that reach CA3 area; from here, part of the fibers leaves hippocampus through fornix, while the so called Schaffer collateral ends in CA1, from here to subiculum and then back to entorhinal cortex again (Davachi et al, 2003).

Entorhinal cortex is connected to parahippocampal gyrus, which is widely connected with temporal, parietal and frontal areas (Figure 15).

![Intrahippocampal circuitry](image)

**Figure 15**
Long Term Potentiation (LTP) is a cellular mechanism essential for memory and learning processes, characterized by a long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously (Cooke et al, 2006). A short high frequency burst of stimuli to every efferent pathway from hippocampus determines increased amplitude of post-synaptic excitatory potentials (Bramham et al, 2008). LTP is induced in CA1 area thanks to Glutamate that is released by Shaffer’s collaterals from CA3.

Glutamate’s receptors are called AMPA and NMDA.

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor, AMPAR, or quisqualate receptor) is a non-NMDA-type ionotropic transmembrane receptor, permeable to Na⁺ but not to Ca²⁺.

Viceversa, N-methyl D-aspartate (NMDA) receptor voltage-dependent activation is a result of ion channel block by extracellular Mg²⁺ ions; this allows voltage-dependent flow of Na⁺ and small amounts of Ca²⁺ ions into the cell and K⁺ out of the cell (Rao, 2007). Ca²⁺ influx is critical for LTP genesis: it activates kinase proteins such as PKA, PKC, CaMK II (Ca²⁺/calmodulin-dependent protein kinase) and MPAK (Mitogen-activated protein kinase), that increase their activity and expression through AMPA receptors phosphorylation.

Also kinases of he Sarcoma Proto-Oncogenic Tyrosine Kinases (Src) family are activated, and they can phosphorylate the same NMDA receptor and increase its sensibility and expression on cellular membrane (Ling et al, 2006).

Moreover, Ca²⁺ induces the liberation of factors such as nitric oxide and arachidonic acid that diffuse towards presynaptic membrane, where they activate second messengers thus increasing Glutamate release therefore maintaining LTP (Bliss et al, 1993).

4.6. DISCUSSION

4.6.1. EPIDEMIOLOGICAL RESULTS

Epidemiological analysis allowed identifying people with cognitive decline, defining frequency and distribution in each isolated population.

Clinical, neuropsychological and laboratory evaluation are not sufficient methods for exactly defining subtype of dementia, and sometimes not even to decide with reasonable certainty that a subject is demented; neuroimaging for example is usually required. In our study therefore an over or underestimation of individuals affected could have been happened.
With these premises, prevalence of possible cognitive decline in the six population examined was distributed as follows: Erto-Casso 14.8%, Sauris 12.8%, S.Martino del Carso 12.6%, Clauzetto 9.4%, Resia 7.1%, Illegio 4.2%. These results seem to point to a higher prevalence in these towns than in general Italian population reported in literature (9% versus 5% respectively).

Single prevalence wise, differences among each population represent an interesting result.

As mean age was comparable in every community, we hypothesized the possible contribution of three main factors:

1. Education differences (among the single communities and relative to general population)
2. Operator-dependent bias
3. Genetic-environmental factors

First point wise, raw scores obtained at MMSE were corrected for age and education. Secondly, we assume that the great prevalence heterogeneity, despite comparable mean age, education level, and same operators, could be almost partly related to genetic-environmental factors, conferring a different predisposition toward dementia.

From analysis of dementia risk factors frequency in subjects with cognitive decline, in comparison to healthy ones, we found out a statistically significant association with comorbidities such as hypertension (OR 2.8), atrial fibrillation (OR 5), and low education (OR 8.2), in accordance with literature data.

4.6.2. GENETIC RESULTS

Genotyping analysis made in this study revealed a possible role of PTPRD gene (9p23-p24.3), coding for a tyrosine phosphatase receptor, on Alzheimer’s disease susceptibility.

Genotyping through GWAS approach was made on 577 subjects (49 cases and 528 controls), using 370000 tagSNPs. Statistical analysis showed a significant association with AD and mixed dementia for 8 SNPs localized on chromosome 9, close to PTPRD gene.

Considering its role within CNS, several studies demonstrated PTPRD gene expression in CA2 and CA3 hippocampal areas, and important role on neuronal plasticity, memory and learning.

As already known, the main cognitive deficit in AD, considering both frequency and entity, is usually related to memory. It often begins as an amnestic syndrome, mainly characterized by difficulty on remembering recent events and learning new information, followed by a progressive loss of previous memories.

Different studies on mice models demonstrated an important role of PTPRD gene in memory and learning processes: knockout mice for this gene showed an impairment of these abilities, which were evident on tasks evaluating spatial learning and memory. Other than that, they
found out an alteration in LTP in CA1 hippocampal area, thus reinforcing the hypothesis of PTPRD gene involvement in memory processes.

PTPRD gene role in AD susceptibility could be verified by replication of this result in comparable but more numerous samples, and by gene sequencing, looking for mutations or specific polymorphisms related to the disease.

An important limit of this study, related to logistic reasons, is that our sample did not have a “pure” phenotype, as cases group was composed by subjects with a clinically possible diagnosis of AD or mixed dementia, without an instrumental confirmation. Even if our diagnostic empiric criteria were studied in order to be as much specific as possible, in order to have a minimal rate of false positive cases, we have no certainty about the possible inclusion of people with other forms of dementia.

For these reasons, a re-evaluation of the population with cognitive decline has been already planned and the use of neuroimaging will be reconsidered to confirm the results.

In the meantime, we decided to analyze if the contribution to our result could come from one or more than one of the communities examined. This could give more strength to genetic analysis, as in the hypothetical case of finding a single contribution on PTPRD result, coming from a genealogic tree with many individuals affected, the possibility of a mendelian way of transmission increases.

All the possible combination and single contribution were performed; statistical analysis revealed the strongest impact factor coming from two populations, Sauris and Erto-Casso. Pedigree analysis was then used to identify if some of individuals affected were also kindred: in Sauris two sisters with dementia were found.

Sequencing of PTPRD gene is now going on, focusing not only on the 21 exons of this gene, but also on the promotor regions.
5. PROJECT n.2: GENE EXPRESSION PROFILING IN
DE NOVO PARKINSON’S DISEASE PATIENTS

5.1. INTRODUCTION:

This study is part of a collaboration project between Neurology Department of University of Trieste and Neurobiology Department of SISSA (Scuola Internazionale di Studi Superiori Avanzati), whose aim is trying to define gene expression profiling in de novo patients with Parkinson’s Disease (PD).

Final goal for the entire project is identifying possible biomarkers for PD, easy to be applied in clinical practice, and building up a platform of clinical and genomic data in order to personalize diagnosis and treatment of what is so far the second most frequent neurodegenerative disorder.

5.1.1. NEED FOR BIOMARKERS IN PARKINSON’S DISEASE

According to Biomarkers Definitions Working Group, a biomarker (biological marker), is a substance used as an indicator of a biological state; it is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarker Definition Working Group 2001). In PD, lack of diagnostic tests that can identify subjects at risk of developing the disease before clinical manifestations, and at the same time helping in differential with other extrapiramidal diseases, emphasizes the need of a specific biomarker, that could potentially:

1. Increase diagnostic accuracy, now reaching 91% (Hughes AJ et al., 2002)
2. Allow a presymptomatic diagnosis, in view of future neuroprotective therapies (Stocchi e Olanow 2003).

Possible biomarkers in PD can belong to three different categories: functional imaging, early symptoms and biochemical/genetic tests.

Considering functional imaging, even if there’s growing evidence about the role of PET with 18-F dopa, I-123 IBZM SPECT and DaT SCAN in detecting presymptomatic alteration of nigrostriatal system (Michell et al, 2004), these modifications do not necessarily mean future development of PD. Actual knowledges do not support the use of these methods as biomarkers for early diagnosis of PD (Ravina et al, 2005).

Also substantia nigra hyperecogenity at Transcranial Doppler (Becker e Berg, 2001) or reduced uptake of metaiodobenzil-guanidine (I-123 MIBG) adrenergic tracer at myocardial
scintigraphy (Braune et al, 2001) are useful tools for PD diagnosis, but they do not reach sufficient sensitivity and sensibility to be used as biomarker.

Considering the frequency of early pre-motor features of PD, described before in the text, an improved accuracy for their detection has been considered, in order to use them as a biomarker. However, these symptoms are aspecific and often too subtle for that purpose.

Biochemical analysis wise, several studies tried to find out possible makers of the oxidoreductive or iron metabolism alterations involved in PD pathogenesis (Jenner et al, 2003; Chinta et al, 2008; Kaur et al, 2004). Summarizing results, there’s plenty of proof about the contribution of cellular homeostasis alteration, reduced antioxidant mechanisms and heavy metal accumulation, in dopaminergic degeneration in PD (Andersen et al, 2004). However, a markers of oxidative stress is not easily applicable in clinical practice, due to lack of specificity and large interindividual variability (Michell et al, 2005).

In conclusion, none of the potential biomarkers so far available satisfies the required characteristics of diagnostic reliability, independence from external factors, easy reproducibility and systematic applicability.

5.1.2. PERIPHERAL BLOOD ALTERATIONS IN PARKINSON’S DISEASE

As previously seen, PD alterations involve not only SNC, but also the autonomic one and other tissues outside nervous system:

- gastro-esophageal damage has been histologically confirmed by LBs findings in myenteric Auerbach’s plexus (Clarke et al, 1998) and immunoreactivity for α-Syn in Meissner’s plexus (Braak et al, 2006);
- orthostatic hypotension affects around 20% of PD patients since the beginning of the disease (Orimo et al, 2007), sometimes in association with cardiac arrhythmias (Barbic et al, 2007);
- urinary symptoms are found in 27-71% of cases (Araki et al, 2000);
- skin sympathetic amyelinic fibers, in particular cholinergic and adrenergic ones, are also damaged, causing sweating and thermoregulation problems in 30-50% of PD patients (Hyraima et al, 2006);
- reduction of dopamine in retinal cells, that could possibly be related to loss of colour discrimination often found in these patients (Harnois et al, 1990);
- in peripheral blood of PD patients, quantitative modifications of T-lymphocytes populations, as well as increased IgG, inflammatory cytokines and pro-apoptotic proteins have been described (Stypula et al, 1996). Remarkable is the finding of early dopamine receptors alterations in lymphocytes: in particular, a study conducted by Nagai et all. in 1996 revealed a reduction in mRNA of D3 dopaminergic receptor in lymphocytes of PD patients, in comparison to sex and
age matched controls (Nagai Y. Et al, 1996), while Barbanti et all. found an increased density of D1-like and D2-like receptors in lymphocytes of de novo PD patients, if compared to other neurologic patients and healthy controls (Barbanti et al, 1999). An intersting recent study demonstrated a reduction of dopamine active transporter DAT in circulating lymphocytes of PD patients, corresponding to reduced synaptic dopamine uptake evidenced by 18F-dopa PET (Caronti et al, 2002).

• Other peripheral blood cells findings of the last years are an hyperexpression of α-Syn in circulating monocytes (Kim et al, 2004), erythrocytes and their precursors (Nakai et al, 2007), and platelets (Li et al, 2002).

In conclusions, peripheral blood cells represent a very important scenario in order to understand molecular alterations associated with PD.

5.1.3. GENE EXPRESSION PROFILING VARIATIONS IN BLOOD CELLS

Transcriptomics is the branch of molecular biology dealing with messenger RNA molecules produced in an individual or population of a particular cell type.

The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA produced in one or a population of cells. Unlike the genome, which is roughly fixed for a given cell line (excluding mutations), the transcriptome can vary with external environmental conditions. Because it includes all mRNA transcripts in the cell, transcriptome reflects the genes that are being actively expressed at any given time. The study of transcriptomics, also referred to as gene expression profiling, examines the expression level of mRNAs in a given cell population, often using high-throughput techniques based on DNA microarray technology.

Obviously, one of the most important applications of transcriptomics is the possibility to find set of disease-related genes, differently expressed in affected subjects in comparison to healthy controls.

These gene-signatures are therefore potential ideal candidates to become diagnostic, prognostic (i.d. treatment response) and stratification (i.d. disease susceptibility) biomarkers.

By definition, these genes should therefore have three essential characteristics:

(1) reproducibility: differential expression has to be accurately reproduced, or replicated, by someone else working independently, being therefore not influenced by environmental or confounding factors;

(2) reliability: expression profiling variations have to be not only sensitive and specific, but also stereotyped in all individuals with the same disease;

(3) concordance: expression profiling variations must have a biological correspondence with
underlying patogenetic molecular mechanisms.

Basic concept for blood gene expression profiling studies in non-hematologic diseases is the possibility that transcriptome in blood cells - easily accessible with non invasive methods - could reflect patognomonic alterations of other tissues, as it already does in response to noxae like myocardial ischemia or glucidic intolerance (Liew et al, 2006; Mohr et al, 2007).

However, among issues of this method are not only the great interindividual variability of transcriptional profiling (Dumeaux et al, 2010), partially related to constitutional factors such as sex, age (Simunovic et al, 2010) and hematologic phenotype (Eady et al, 2005), but also its being extremely sensitive to external factors such as physical exercise (Connolly et al, 2005), circadian rhythms (Zhu et al, 2010), food intake (Leonardson et al, 2010) or tobacco smoke (Lampe et al, 2004).

Considering gene-signatures in diseases, there’s evidence of different subtype specificity and therapy responsiveness in hematologic tumors (Ebert et al, 2004) and encouraging results for mammalian, colon and renal carcinomas (Smirnov et al, 2005; Li Y. et al, 2006), but also for non oncologic diseases like arthritis, schizophrenia, Alzheimer’s Disease and others (Mohr et al, 2007).

In conclusion, there’s growing evidence pointing to blood cells as a source of biomarkers.

5.1.4. GENE EXPRESSION PROFILING IN NEUROLOGY

Peripheral blood cells share with neurons the same gene pool, and the transcriptome concordance is over 80% (Mohr et al, 2007).

One of the first studies on this topic was made on mice in 2001: gene expression profiling was very sensible to different SNC noxae, such as stroke, hypoglycemia, epilepsy and hypoxia (Tang Y. Et al, 2001).

Similar results were then replicated in humans, at the beginning in genetic diseases like neurofibromatosis type 1 (Tang Y. et al, 2005) and Huntington Disease. In the latest case, 322 transcripts were significatively \( p<0.0005 \) altered in affected subjects in comparison to healthy controls; in particular, a subgroup of these mRNA was able to effectively discriminate between controls, asymptomatic carriers and affected subjects. The same pool of genes was found to be differently expressed in post-mortem caudate samples, confirming that transcriptome in peripheral blood is strictly related to patogenetic events in basal ganglia (Borovecki et al, 2005).

Parkinson Disease wise, Sunada et al. in 1998 revealed that all genes responsible of monogenic PD are actively expressed in blood cells (Sunada et al, 1998) and recent studies on lymphocytes and platelets of PD patients showed subtle alterations in genes related to dopamine
synthesis and signaling, and in regulation of mitochondrial function, two pathways known to be involved in PD pathogenesis (Petrozzi et al, 2001; Yoshino et al, 2002).

In another gene expression profiling study published in PNAS (Scherzer et al, 2007), a set of 22 genes were differently translated in 50 early onset PD patients and 55 matched healthy controls; 8 out of those genes are good candidates to be a possible PD stratification biomarker, as this multigenic marker is associated with an increased risk of developing the disease (ODDS 5.7, \(p < 0.005\)).

Real time PCR on one of those 22 genes identified was made in peripheral blood of 13 de novo PD patients and 29 treated patients (Shadrina et al, 2010). That gene, suppressor of tumorigenicity ST-13, encodes for a protein of ubiquitin-proteasomic system that also mediates bond between heat-shock-proteins HSP70 and HSP90. The former protein intervenes in \(\alpha\)-syn folding and suppression of citotoxicity.

Unfortunately, difference of ST-13 expression in healthy matched controls did not reach statistic significante, and therefore cannot be used as a diagnostic biomarker.

Finally, Shehadeh et al. in 2010 performed an extensive analysis on three publicly available PD datasets generated from different biological sources: a neuroblastoma line, substantia nigra from PD patients and controls, and lymphocytes from PD patients and controls. They found that the RNA splicing gene SRRM2 (sereine/arginine repetitive matrix 2), was the only gene differentially upregulated among all the three PD experiments. SRRM2 expression was not changed in the blood of other neurological-diseased patients versus the healthy controls. The consistent dysregulation of the RNA splicing factor SRRM2 in two different PD neuronal sources and in PD blood but not in blood of other neurologically diseased patients makes SRRM2 a strong candidate gene for PD and draws attention to the role of RNA splicing in the disease. In addition, they reported novel information about hundreds of genes with significant alternative splicing (differential exonic expression) in PD blood versus controls.

This study can be considered a paradigmatic example of advantages of gene expression profiling in PD diagnosis and pathogenetic mechanisms.

One of the most important limits of all studies on PD derives from the choice of patients with monogenic forms of disease or with sporadic but long lasting and on treatment forms. Obviously, none of these groups represents the ideal model to study molecular events of a typical idiopatic PD, or to pick up a biomarker for early diagnosis.

This is the starting idea of our project, that is to analyze a group of de novo idiopatic PD patients, without any pharmacological treatment.
5.2. OBJECTIVES OF THE STUDY

Three are the main objectives of this study:

1. Developing a common platform of clinical, neuroimaging and genomic data of patients with sporadic PD;
2. Identifying possible correlations between phenotype and genotype, that could help in a more personalized diagnostic and therapeutic approach to the patient;
3. Evaluating the contribution offered by genomics in finding early diagnostic biomarkers.

For these purposes, the study briefly describes procedures and methods followed for clinical and genomic data collection of 40 de novo PD patients and 20 healthy controls, matched for sex, age, and environmental influence; we will then discuss the main functional pathways of genes differently expressed in the two groups; finally, we will elaborate on these preliminary results possible hypothesis of clinical-genomic correlation.

5.3. PATIENTS AND METHODS

Study group was composed of patients evaluated at Neurology Department of University of Trieste between April 2007 and April 2010.

On admission, all patients underwent the same diagnostic protocol for confirmation of suspected PD (Table 5).

<table>
<thead>
<tr>
<th>CLINICAL EVALUATION</th>
<th>INSTRUMENTAL PROCEDURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familiar, personal and past medical history, history of present illness</td>
<td>TC/RMN cerebrale</td>
</tr>
<tr>
<td>General physical examination</td>
<td>Transcranial Doppler (TCCD)</td>
</tr>
<tr>
<td>Neurologic examination</td>
<td>SPECT with presinaptic tracer (DaTSCAN)</td>
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<tr>
<td>Routine Laboratory Tests</td>
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Table 5 : Clinical-instrumental diagnostic protocol

For each PD patients, two internationally recognized motor scales and a cognitive screening were applied: *Unified Parkinson Disease Rating Scale* (UPDRS) and *Hoehn-Yahr Rating Scale* (H&Y), and Mini Mental State Examination (MMSE) - as cognitive decline was an exclusion criteria.
**UPDRS** is a very common scale for PD evaluation, divided in four sessions:

1. Mentation, behavior and mood (4 items)
2. Activities of daily living (13 items)
3. Clinician-scored motor evaluation (Columbia Disability Scale, 14 items)
4. Complications of therapy (11 items)

With the exception of seven items with dichotomic answer/score (No=0, Yes=1), all other items can be scored from 0 (perfectly normal) to 4 (severely compromised). Total score is obtained as the sum of the first three partial scores, and is directly related to disease severity (0-176).

**Hoehn-Yahr** (Goetz et al, 2004) is a very useful and common clinical instrument for evaluation of disease severity and its progression over time

- Stage 0: No signs of disease.
- Stage 1: Unilateral symptoms only.
- Stage 1.5: Unilateral and axial involvement.
- Stage 2: Bilateral symptoms. No impairment of balance.
- Stage 2.5: Mild bilateral disease with recovery on pull test.
- Stage 4: Severe disability, but still able to walk or stand unassisted.
- Stage 5: Needing a wheelchair or bedridden unless assisted.

**MMSE** has already been described in the previous project.

Inclusion criteria were: (1) diagnosis of PD according to UKPDS Brain Bank criteria (2) age >30 years (3) symptoms of PD lasting less than 2 years. Exclusion criteria were: (1) important cerebrovascular disease involving basal ganglia on neuroimaging; (2) symptoms of Parkinson-plus diseases (e.g. Multisystem Atrophy, Progressive Supranuclear Palsy) (3) symptomatic Parkinsonism; (4) treatment with neuroleptic drugs in the last 6 months; (4) cognitive decline.

We therefore selected 40 subjects affected by typical idiopathic PD that were compared with 20 healthy controls, matched for sex and age, sharing with patient a common environmental background (e.g. spouses).

During the first medical visit, each subject received detailed information about methods and purposes of the study, and signed an informed consent.

The research project respected all the Italian laws and rules in terms of privacy, biological samples collection and storage, and genomic test.

Blood samples collected at Neurology Department were sent to SISSA Neurogenomic Laboratory, where they were properly stored; quantitative and qualitative total RNA analysis and total RNA extraction were performed.

Samples were then prepared and processed through Oligonucleotide Microarrays (Affymetrix GeneChips platform) in order to have gene expression profiling for each subject.
Following processes of filtration and clustering of raw data led to identification of a pool of genes differently expressed in the two groups. These statistically significant results represent therefore a promising field where to look for a possible PD biomarker, but confirmation studies using different technologies (e.g. \textit{realtime PCR}) are needed.

For this reason, detailed characteristics of these genes - at the moment under verification protocols - won’t be discussed in this thesis.

\textbf{5.4. EXPERIMENTAL PROTOCOL}

Blood collection for gene expression profiling analysis was made.

As procedure control, 5 patients underwent a second blood draw, after one week from time 0. Each blood draw was made around 9 a.m., and with fasting patient.

Samples were collected and stored using \textit{PAXgene Blood RNA System} that guarantees a better intracellular RNA stabilization, as it normally is very prone to lytic degradation. A special additive inside the \textit{PAXgene Tube} limits genetic induction, frequently observed \textit{in vitro} and potentially responsible of quantification errors \textit{in vivo}.

For each patient we collected 20 ml of peripheral venous blood, subdivided in eight 2.5 ml \textit{PAXgene Blood RNA}, that were slowly turned upside down 8-10 times each, in order to let blood perfectly mix with solution buffer; tubes were then kept in vertical position at temperature of 20-25°C for 4-6 hours, and then at +4°C for a maximum of 24 hours.

Finally, samples were sent at SISSA Neurogenomic laboratory and cooled down at -20°C for hours and then stored at -80°C till extraction procedures.

TotalRNA extraction was made using Quiagen \textit{PAXgene Blood RNA kit} and protocols: in brief, each PAXgene Blood RNA tube is slowly warmed up at 18-25 °C in 15-18 hours, then centrifugated for 10 minutes at 3000-5000 g, to make nucleic acids precipitate. Obtained pellet is therefore treated with proteolytic enzymes, suspension buffer and RNAse-free washing solution to remove all DNA molecules. After a second centrifugation, washing and filtering procedures, totalRNA was finally extracted.

Quantitative totalRNA analysis was made with ND-1000 UV-Visible light Spectrophotometer. The newly developed sample retention system allows for rapid (10 seconds) spectrum measurement of DNA, RNA, protein or dye using small samples volumes. Qualitative totalRNA analysis and separation of its different components was made using capillary electrophoresis (Agilent 2100 Bioanalyzer).

Further steps were cDNA preparation through \textit{Ovation RNA Amplification System V2} (NuGen) and Oligonucleotide Microarrays with Affymetrix GeneChips, therefore obtaining gene expression profiling for each subject.
5.5. RESULTS

5.5.1. CLINICAL FINDINGS

We enrolled 40 patients, with male/female ratio of 22/18, mean age 66.9 ± 6.3, ranging from 51 to 77. Controls were 20 healthy subjects (M/F=11/9), matched for age, sex and environment.

Relevant clinical characteristics of the 40 PD patients are summarized in the Table 6; we do not describe clinical findings of controls as almost none of them did show pathological signs or symptoms, and they are not relevant to the purposes of this study.

On presentation, tremoric-bradikinetic-hypertonic phenotype was the most frequent (55%, n=22), followed by the hypertonic-bradikinetic (27.5%, n= 11) and the tremoric one (17%, n=7).

From history of present illness, most patients (60%, n=24) complained of upper limb tremor as symptom of onset, often accompanied by bradikinesia (63%).

20% of patients (n=8) reported bradikinesia as first symptom of the disease, while only 5% (n=2) of patients complained of aspecific aching and recurrent back pain at onset.

On neurological examination, 39% (n=12) of patients presented gait difficulties, while a noticeable reduction of synkinetic movements of the upper extremities was found in 80% (n=32) of patients.

Non-motor symptoms like anxiety and depression disorder, hypoosmia and sleep difficulties were reported by 22.5% (n=9), 15% (n=6) and 12.5% (n=5) of patients respectively.

On general examination, 30% (n=12) of PD patients had hypertension (Pad >110 or Pas >140), in the 84% of cases in comorbidity with other cardiovascular risk factors such as dislipidemia or diabetes type 2.

Routine laboratory test wise, 22.5% (n=9) and 35% (n=14) of cases had respectively high fast glucose (>126 mg/dL) and hypercholesterolemia (>240 mg/dL).

Not statistically significant differences in cardiovascular risk factors between cases and controls were found (data not shown).
Table 6: relevant motor and non-motor clinical features of PD patients

<table>
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<th>N</th>
<th>%</th>
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<tr>
<td><strong>Total patients</strong></td>
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<td>100</td>
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<tr>
<td><strong>PHENOTYPE</strong></td>
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<tr>
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<td>7</td>
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<tr>
<td>Hypertonia-Brakinesis</td>
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<td>27.5</td>
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<tr>
<td>Tremor-Hypertonia-Brakinesis</td>
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<td>55</td>
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<tr>
<td><strong>MOST AFFECTED SIDE</strong></td>
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<td>22.5</td>
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<tr>
<td>Left</td>
<td>15</td>
<td>37.5</td>
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<tr>
<td><strong>GAIT DIFFICULTIES</strong></td>
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<tr>
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<td>30</td>
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<tr>
<td>Absent</td>
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<td>70</td>
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<td><strong>SYNKINETIC MOVEMENTS</strong></td>
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<tr>
<td>Absent</td>
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<td><strong>CAMPTOCORMIA</strong></td>
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<tr>
<td><strong>MICROGRAPHIA</strong></td>
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</tr>
<tr>
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<td>90</td>
</tr>
<tr>
<td><strong>ANXIETY-DEPRESSION</strong></td>
<td></td>
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<tr>
<td>Present</td>
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</tr>
<tr>
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<td><strong>HYPOSOMIA</strong></td>
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<tr>
<td><strong>SLEEP DISORDERS</strong></td>
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<tr>
<td>Present</td>
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<td>Absent</td>
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<td>87.5</td>
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</table>
UPDRS scores, in particular parts II and III focused on ability of daily living and motor symptoms, are summarized in Figure 16. Mean score obtained was 15/160.

Hoehn-Yahr scores showed that disease severity was usually mild in our patients: 55% (n=22) had unilateral symptoms only (Stage I), 42.5% (n=17) bilateral symptoms without balance impairment (Stage II), only one patient (2.5%) was at Stage III (Balance impairment, mild to moderate disease, physically independent).

**Figure 16: Distribution of UPDRS II-III scores among patients**

5.5.2. NUCLEAR MEDICINE FINDINGS

Table 7 summarizes results obtained from $^{123}$I-DaTSCAN SPECT and their concordance with clinical findings.

During images acquisition (~30 minutes), 30% of cerebral activity of radioactive isotope is related to its striatal uptake, and increases in 3 hours till a pick, then followed by a 6 hours’ plateau. In PD patients, that pick is reached in 2-2.5 hours. Final analysis is both qualitative (in relation to head of caudate and putamen images, see figure below) and quantitative, as a ratio of bilateral striatal/occipital-cortex uptake is calculated.

As a rule, a pathological ratio is considered when uptake is reduced >2 SD in comparison to normal value in healthy controls (4±0,5).
Figure 17. 123I-Ioflupane SPECT (DaTSCAN) of a normal subject (left) and of a PD patient (right), where a typical pattern of bilateral asymmetric reduced putamen uptake is observed.

In each patient a functional alteration of nigrostriatal dopaminergic system was confirmed by a typical pattern of bilateral asymmetric reduced tracer uptake (Figure 17), which in 27.5% (n=11) of patients was quantitatively detectable.

Concordance between side of more affected limbs and controlateral more reduced uptake was 93%, with only three subjects having more reduced uptake ipsilaterally to side affected. In general, pattern of DaTSCAN alteration was directly related to disease severity, according to literature evidence.

**TABLE 7 : Semiquantitative values of DaTSCAN-SPECT in our PD patients**

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<th>BG DX/Occ</th>
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<td>n.v. 4±0.5</td>
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<tr>
<td>2.84</td>
<td>-1.66</td>
<td>2.29</td>
<td>-2.21</td>
</tr>
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</table>

n.a. = not available

5.5.3. GENE EXPRESSION PROFILING ANALYSIS

Transcriptome data obtained with GeneChip Affymetrix then underwent a statistical analysis procedure apt to detect signal intensity (Cell Intensity), which is directly related to transcriptome quantity, and bond avidity between probe and transcriptome (Detection Call).

Cell Intensity and Detection Call analysis were performed by Affymetrix GeneChip operating Software (GCOS). Row data were then normalized and background-adjusted using a particular
package of the statistical software “R” called Bioconductor, and Robust Multi-Array Average Normalization (RMA) algorithm.

All data were then “filtered” in order to keep only patterns of gene expression profiling with a statistically significant different expression in the two groups (SAM – Significant Analysis of Microarray).

Trascriptomes thus obtained finally underwent a functional classification process through Gene Sets Enrichment Analysis (GSEA) [http://www.broadinstitute.org/gsea](http://www.broadinstitute.org/gsea).

As expected, comparison of gene expression profiling between first and second blood samples in five patients did not show any statistically significant difference.

Preliminary analysis of transcriptomes identified an interesting pool of genes differently expressed in cases and controls: data clusterization recognized among them specific subsets of genes belonging to the same functional pathways.

Graphic representation of results (Figure 18) shows on the y-axis all subjects codes (double blinded), on x-axis probesets for recognition of oligonucleotides sequences in specific genes. Cromatic representation used indicates in red probes of over-expressed genes, in green the suppressed ones, after comparison between cases and controls.

Finally, GSEA allowed to better characterize functional processes some of the identified genes belong to; preliminary results seem to point towards a correlation between PD and the following pathways:

- Hypoxia
- Axonal guidance
- Calcium signaling
- Inflammation
- Glycosphingolipid metabolism
- Neurotransmitters pathways
- Diabetes
- Neurodegenerative disease
Figure 18: Cluster Analysis of transcriptomes in 40 PD patients and 20 healthy controls, matched for age, sex and environmental factors
6. CONCLUSIONS

6.1. GENETIC PARK PROJECT FVG

Complex diseases such as hypertension, diabetes, tumors and neurodegenerative diseases including dementia are a very common condition whose pathogenesis is determined by interaction between genetic and environmental factors, and are therefore defined as multifactorial diseases.

Alzheimer Disease wise, traditional dichotomization in early-onset familial AD (EOFSD) and late-onset AD (EOAD) is overly simplistic as there are cases of early-onset AD without evidence of Mendelian transmission while, conversely, LOAD - sometimes referred to as “sporadic” AD - is frequently observed with a strong familial clustering, sometimes resembling a Mendelian pattern. Moreover, up to 60%–80% of this form of AD is genetically determined. While EOFAD is caused by rare and highly penetrant mutations in three genes (APP, PSE1, PSEN2), the genetics of LOAD is more complex. According to current thinking, susceptibility for LOAD is conferred by numerous genetic risk factors (also called susceptibility genes), characterized by relatively high frequency but low penetrance and therefore small effect size. Upon that, environmental and epigenetic factors play an important role in determining an individual’s risk, although the precise nature and mechanisms underlying this nongenetic component remain largely obscure, in part because they are difficult to assess experimentally.

For this reason, genome-wide association studies (GWAS) applied to isolated populations seems to be the best combined approach to provide further inside into pathogenesis of neurodegenerative diseases, and AD in particular.

A Genome–wide association study (GWAS) is a study made on the entire genome, in order to analyse its genetic variations. It consists of evaluation of the genome in different individuals, obtained using the highest possible number of genetic markers without any a priori gene knowledge and/or selection.

A GWAS implies the choice of cases and controls, the selection of SNPs spread along the all genome, and their genotypization. Its objective is identifying genetic associations with observable traits or the presence/absence of a disease/condition.

Isolated populations, characterized by hundreds of years of isolation and by the maintenance of a traditional life style, make easier not only recognizing the influence of environmental variability on phenotypes, but also studying the homogeneous genetic background; inbreeding, typical of small communities, reduces genetic heterogeneity and increases homozygosis, providing greater power for detection of susceptibility genes.

As a matter of facts, general reduction in genetic and environmental variation as well as the availability of confined, well-documented extended pedigrees, results in an increase in statistical power to identify genes, particularly in terms of the ability to map genetic disease loci.
Our study was born as part of the multidisciplinary “Friuli Venezia Giulia Genetic Park” project, whose purpose is to discover possible new susceptibility genes for multifactorial diseases. It took place in six small isolated communities of Friuli Venezia Giulia.

Goals of the neurology team were specifically the evaluation of dementia prevalence, presence and association with known dementia risk factors, and possibly to find out new genes predisposing to AD and mixed dementia, that are nowadays-relevant social health issues in developed countries.

Greater knowledge about these genes, as well as comparison with styles of life and environmental variables have potentially a very strong impact not only on public health, but most of all on better understanding of AD pathogenesis.

This aspect could contribute to studying and synthesizing new drugs apt to prevent, specifically treat or block AD progression; it could also focus on environment interventions to be done.

Considering the neurological session of this study, evaluations were conducted on 920 subjects, older than 40 years, who all underwent accurate history collection, as well as neurological examination and neuropsychological screening, in order to pick out people possibly affected by dementia. 89 individuals were in this way selected that means a greater percentage of total population of these villages, if compared to literature data concerning Italian population. All information collected about personal history and past/present medical issues allowed us to identify an association between dementia and some known risk factors, in particular hypertension and atrial fibrillation, as well as between dementia and low education level.

Genetic analysis, through GWA method, was conducted on a sub-population of 49 subjects, with a clinical diagnosis of possible AD or mixed dementia. Criteria were purely clinic, but we think they are sufficiently strict to be specific enough (the least false positives).

These 49 subjects were then compared to 528 controls with no cognitive decline, and results indicated a possible role of PTPRD gene (chromosome 9) in AD susceptibility.

In mice, PTPRD gene is involved in formation and maintenance of synapses, by promoting regeneration and axonal transport. It is therefore an important regulator of neuronal plasticity and is implicated in memory and learning processes.

In humans (Uetani et al, 2000) PTPRD seems to have an inhibitory role in LTP, probably through a pre- and post-synaptic mechanism in hippocampal CA1 and CA3 areas, but no other did confirm or replicate this interesting result.

We therefore decided to analyze the contribution played by each community or combination of communities to this result; this could give more strength to genetic analysis, as in case of a single or limited contribution on PTPRD result, accurate study of pedigrees could highlight a possible mendelian transmission.

From all the combinations performed among communities, statistical analysis revealed the
strongest contribution from two populations, Sauris and Erto-Casso. Pedigree analysis was then helpful in identifying if some of individuals affected were also kindred: in Sauris two sisters with dementia were found.

Sequencing of PTPRD gene is now going on, focusing not only on the 21 exons of this gene, but also on the promotor regions.

GWAS have substantially reshaped the world of LOAD genetics during the course of only three years. Currently, the most promising findings - already confirmed - relate to the identification of variants near BIN1, CLU, CR1, and PICALM. Other GWAS loci, such as EXOC3L2 and MTHFD1L, still need to be replicated.

Interestingly, nearly all of the newly reported GWAS loci have been linked to Aβ metabolism, in particular Aβ aggregation and clearance from the brain.

In the landscape of GWAS, this study therefore presents some relevant and unique characteristics:

(1) This is to our knowledge the first report of PTPRD involvement in dementia in humans;
(2) No other GWAS has previously found a correlation between AD and PTPRD (see AlzGene);
(3) This is to our knowledge the first GWAS in demented patients randomly chosen in isolated populations, not recruited in specific medical setting.

Considering last point, two recent GWAS were performed as part of the Genetic Research in Isolated Populations (GRIP) program, which was conducted in a isolated population from the southwestern area of The Netherlands.

In cooperation with local general practitioners, neurologists and nursing home physicians, researchers asked patients with any dementia syndrome and their relatives to participate.

Sleegers et al. (2004) ascertained 191 patients comprised 122 probable LOAD and 17 EOAD, and 22 with possible AD, 10 patients with vascular dementia, nine with Lewy body dementia and six with frontotemporal dementia. Their data showed a strong familial clustering of various forms of dementia, EOAD in particular; although 14% of LOAD had evidence of autosomal dominant disease, consanguinity was found in three of them suggesting a recessive or polygenic model underlying the trait. A high percentage of late-onset Alzheimer's disease could be explained by APOE4, but 55% of its origin was still unknown.

With the same methodological approach, Liu et al. (2007) conducted a genome screen of 103 patients with LOAD and their 170 closely related relatives who were ascertained in an isolated population. As in the study above described, patients were recruited with contribution of local general practitioners and neurologists.

Authors confirmed two previously well-described linkage regions for late-onset AD on chromosomes 1 and 10, and for the first time showed significant linkage with 3q23 markers.
NINCDS-ADRDA clinical diagnostic AD criteria were used in these two studies, as well as in ours, but they achieved a better diagnostic accuracy, thanks to previously made diagnosis and clinical/instrumental exams, and the possibility of reviewing medical records. However, the choice of ascertain only a pre-defined subpopulation of the entire community, can represent a bias in comparison to random recruitment, and be therefore not completely representative of the total population object of the study.

If the random choice of patients can be considered a strength of our study, there are however two important weaknesses, that may have influenced our results.

First of all, even if our diagnosis was based on internationally recognized diagnostic criteria, environmental conditions did not allow clinical diagnosis to be confirmed by proper neuroimaging, as usually required in clinical and research settings. To partially overcome this aspect, we therefore decided to affine our patients’ selection by means of increasing specificity (but also lowering sensitivity), through further accurate analysis of vascular comorbidities, neurological examination and performances at neuropsychological screening. Even if not standardized, this approach is commonly applied also in clinical practice.

The second important weakness is the small sample we were able to collect.

While genomewide screening has many advantages, massive multiple testing is a critical issue and substantially more rigorous criteria are required to declare an association as being “significant” on an experiment-wide level. Several p values thresholds to declare genomewide significance have been proposed, but optimal population matching and high-quality genotype will give reliable and useful results in presence of a large enough sample size — GWAS is a numbers game where the allele frequencies, effects sizes (the unknown) and the population size, and quality of data determine its success.

Future perspective wise, PRPRD gene sequencing will be the closer step, hopefully to pick up possible mutations or polymorphisms, followed by result replication in more numerous populations matched for age, education and suspected diagnosis.

We are therefore planning to recruit subjects with a definite clinical diagnosis of AD and AD plus cerebrovascular disease, and perform genotypization for those 8 SNPs found to be relevant in our study.
6.2. GENE EXPRESSION PROFILING IN de novo PD PATIENTS

According to results of gene expression profiling in PD patients, some specific pathways seem to be related to PD pathological processes, in particular: hypoxia, axonal guidance, calcium signaling, inflammation, diabetes, glycosphingolipid metabolism, neurotransmitters pathways, neurodegenerative diseases.

Only some of them have been extensively reported in literature.

Inflammation, diabetes mellitus and neurodegeneration belong to this category, as already described in a recent study by Moran et al. in 2008. After extensive brain tissue-based analysis, they provide the complete list of 892 highly dysregulated PD nigral genes derived from a brain tissue-validated whole genome expression microarray data set. They revealed significant up-regulation of substantia nigra genes in PD which belong to “peripheral networks”, as they have known biological associations with cancer, diabetes and inflammation. Interestingly, this includes major ‘hub’ genes such as p53. This is of note as p53 forms part of a molecular network that integrates tumour suppression and ageing; DJ-1 is another cancer- and Parkinson-related gene, and both parkin and PINK1 might be tumor suppressor genes.

Another important pathway emerged from our study, that is glycosphingolipid metabolism; there’s growing interest in literature on this topic, from correlation Gaucher/Parkinson diseases (Ron et al, 2010) to neurodegenerative disorders where condensed membrane domains formed by sphingolipids and cholesterol are privileged sites for the binding and oligomerization of amyloidogenic proteins (Fantini et al, 2010).

The problem of defining what causes PD at a system level has become more complex with the recent finding that disease-relevant genes (e.g. those related to inflammation) may reside at the periphery of disease networks.

A part from the importance of clarifying neuropathological mechanism underlying PD, we think that our study and results could be a possible source of potential biomarker for Parkinson’s Disease.

Further studies and replication of our findings are obviously mandatory for their validation, but this work has a really relevant and new aspect, that is the choice of typical idiopathic de novo PD patients. We consider this an excellent model to look for molecular pathways alterations ab inizio, to be used as early diagnostic marker of the disease, as prognostic factors according to their modification with time and medications, and also as a starting point for developing new therapeutical strategies.

Finally, procedures and methods used in this protocol can guarantee a standardized, qualified and reliable approach, needed for Good Laboratory Practicies (GLPs) and Good Clinical Practicies (GCPs).
If our results will be confirmed, future perspectives would be the evaluation of:

- how gene expression influences PD clinical expression
- how different treatments (L-Dopa, Dopamine Agonists, MAO-B Inhibitors) can influence gene expression
- how gene expression influences treatment response (which is the best therapy for each patient?)
7. References:


64. Di Mauro S, Schon E.A. Mitochondrial disorders in the nervous system. *Annual Review of Neuroscience* 2008; 31: 91–123


70. Ebert B.L. and Golub TR, Genomic approaches to hematologic malignancies *Blood*, 2004; 104: 923-932.
71. Elia J, et al. Rare structural variants found in attention deficit hyperactivity disorder are preferentially associated with neurodevelopmental genes. *Mol Psych* 2010; 15:637-646


114. Ishihara et al. Estimated life expectancy of Parkinson’s patients compared with the UK population. J Neurol Neurosurg Psychiatry 2007; 78(12):1304-1309


147. Ling D. Protein kinase Mζ enhances excitatory synaptic transmission by increasing the number of active postsynaptic AMPA receptors. *Hippocampus* 2006; 16:443-452


166. MCKeith I. Dementia with Lewy bodies. Handbook of Clinical Neurology 2007; 84:531-548


228. Schormair B, *et al.* PTPRD (protein tyrosine phosphatase receptor type delta) is associated with restless legs syndrome. *Nat Genet* 2008; 40:946-948


242. Solfrizzi V, Colacicco A. Macronutrients, aluminium from drinking water and foods, and other metals in cognitive decline and dementia. *J Alzheim Disease* 2006;10


264. Veeriah S, et al. The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. *PNAS* 2009;106: 9435-9440


266. Ward A, et al. Describing cognitive decline of patients at the mild or moderate stages of Alzheimer's disease using the Standardized MMSE. *Int Psychogeriatr* 2002; 14:249-58


