Chapter 1

Mutations of PARK Genes and Alpha-Synuclein and Parkin Concentrations in Parkinson's Disease

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1. Introduction

Parkinson's disease (PD) is a chronic and progressive neurological disorder characterized by resting tremor, rigidity, and bradykinesia, affecting at least 2% of individuals above the age of 65 years. Parkinson's disease is a result of degeneration of the dopamine-producing neurons of the *substantia nigra*. Available therapies in PD will only improve the symptoms but not halt progression of disease. The most effective treatment for PD patients is therapy with L-3,4-dihydroxy-phenylalanine (L-dopa) [Olanow, 2008].

It is now believed that the cause of PD, are both environmental and genetic factors. During the last two decades, there has been breakthrough progress in genetics of PD. It is known that genetic background of PD is in mutations a number of pathogenic genes PARK, e.g. *SNCA, PRKN, UCHL1, DJ-1, PINK1, ATP13A2*, and *LRRK2* (Polrolniczak et a., 2011, 2012). In 2001, Shimura et al. first described the presence in the human brain complex containing Parkin with the glycosylated form of the alpha-synuclein (ASN, alpha-SP22). Moreover, the study by Dorszewska et al. (2012) has been shown, that in the PD patients increased plasma level of ASN was associated by the decreased of Parkin plasma level. It has also shown that configuration: increased plasma level of ASN and decreased of Parkin concentration was associated with earlier onset of PD. It seems that in PD genotypic testing of PARK mutations and analysis of their phenotypes (e.g. ASN, Parkin) may be diagnostic agents for these patients.

2. Mutations in *PRKN, SPR* and *HTRA2* genes and polymorphism of NACP-Rep1 region of *SNCA* promoter in the patients with Parkinson's disease

During the last two decades, there has been breakthrough progress in genetics of PD. Currently it is known that genetic background of PD is heterogeneous and mutations in a number of pathogenic genes (e.g. *SNCA, PRKN, UCHL1, DJ-1, PINK1, ATP13A2*, and *LRRK2*) have been described as associated with familial (FPD) or as genetic risk factors increasing the risk to develop of sporadic PD (SPD). Some of these genes (like *SNCA* and *PRKN)* are fairly well understood while the others (like *SPR* and *HTRA2*) are still little known (Corti et al., 2011).

Monogenic forms, caused by a single mutation in a dominantly or recessively inherited gene, are well-established. Nevertheless, they are relatively rare types of PD and account for about 30% of the FPD and 3–5% of the SPD cases. Although 18 specific chromosomal locus (called *PARK* and numbered in chronological order of their identification) have been reported as more or less convincingly related to FPD (Klein & Westenberger, 2012), the majority of PD cases are SPD (only about 10% of patients report a positive family history) [Thomas & Beal, 2007] while the results of the studies of SPD genetic are still ambiguous and divergent in different ethnic origin (Klein & Schlossmacher, 2007; Lesage & Brice, 2009). Few studies (e.g. Abbas et al., 1999; Gilks et al., 2005; Guo et al., 2010; Mellick et al., 2005; Trotta et al., 2012) suggest a strong correlation of genetic factors with an increased risk of SPD development, while the other reports are contradictory (Chung et al., 2011; Spadafora et al., 2003). However, genome-wide association studies have provided convincing evidence that polymorphic variants in some genes contribute to higher risk of SPD (Gao et al., 2009). Moreover, it is suggested that the etiology of PD is multifactorial, which probably results from coocurence of genetic and environmental factors (Klein & Westenberger, 2012).

Summarizing, from the existing studies reported, it is not yet clear how common mutations in few genes, including: *PRKN*, *HTRA2*, *SPR* and *SNCA* genes contribute to idiopathic PD (Nuytemans et al., 2010). Finally despite previous reports, significance of these genes mutation and polymorphism in pathogenesis of PD (especially SPD) is not clear and is still debated, mainly because of discrepancy of studies results and variance between different ethnic populations. To clarify these issues, more data of genetic analysis are needed while there were only a few reports of genetic studies of PD in Polish populations (Bialecka et al., 2005; Koziorowski et al., 2010). Moreover, little is understood about putative director functional interactions between the genes that cause PD, and a single pathway unifying these factors has not been confirmed (Bras et al., 2008; Brooks et al., 2009; Klein et al., 2005).

2.1. Polymorphism of NACP-Rep1 region of *SNCA* promoter in the patients with Parkinson's disease

SNCA gene, encoding ASN, was first gene describing as related with PD. Missence mutations and multiplications of this gene, generally have been described as related with FPD (Kruger et al., 1998), however *SNCA* duplications were also reported in SPD (Abeliovich et al., 2000;

http://dx.doi.org/10.5772/57107

Ahn et al., 2008; Liu et al., 2004; Nishioka et al., 2009; Nuytemans et al., 2009). Therefore, it have been suspected that not only mutations in the *SNCA* gene, but also other factors affecting the expression of ASN may contribute to the PD manifestation including, SPD.

The study by Chiba-Falek et al. (2006) has shown that the region NACP-Rep1 of *SNCA* gene promoter, there is the polymorphic region differenting in dinucleotide repeats count and affecting the level of ASN expression. Moreover, it has been shown that polymorphism of NACP-Rep1 region in promoter of *SNCA*, are associated with an increased risk of SPD in some population like: German, Australian, American and Polish, but the other multi-population studies have observed no association or reported an inverse association between the risk allele and PD (Farrer et al., 2001; Kruger et al., 1999; Maraganore et al., 2006; Polrolniczak et al., 2012; Tan et al., 2003).

Region NACP-Rep1 contains dinucleotide repeats (TC)x(T)2(TC)y(TA)2(CA)z, which may vary both the number of repeats, and include substitutions of nucleotides. However, it has been proven, that a change in the length of the NACP-Rep1 region more than substitutions, affects the regulation of the expression of ASN (Fuchs et al., 2008; Mellick et al., 2005; Tan et al., 2003). As the most common in humans it has been described five alleles of NACP-Rep1 of the *SNCA* gene promoter: -1, 0, +1, +2, +3. Generally in the European population the most frequently was allele +1 of NACP-Rep1. It has been also shown, that the allele 0 of NACP-Rep1 region in *SNCA* promoter is two pairs shorter than allele +1, allele -1 respectively, shorter by 4 bp however alleles 2 and 3 are longer by 2 and 4 bp.

Functional analysis on the two most common NACP-Rep1 alleles +1 and +2 suggested that the +2 allele is associated with an up-regulation of *SNCA* expression, whereas the +1 variant shows reduced gene expression (Chiba Falek & Nussbaum, 2001; Cronin et al., 2009). In addition, allele +1 of the region NACP-Rep1 of *SNCA* promoter, containing 259 bp, significantly reduces the risk of PD in the population of Europe, America and Australia (Fuchs et al., 2008; Maraganore et al., 2006; Tan et al., 2000) while another study failed to replicate the finding in population of Japan, Singapore and Italy (Spadafora et al., 2003; Tan et al., 2003).

Nerveless, although protective effect of allele +1 rather not currently subject to discussion, but for alleles 0, +2 and +3 it has been suggested both no impact, as well as increasing the risk of PD, and even sometimes the protective action (Maraganore et al., 2006; Spadafora et al., 2003; Tan et al., 2000; Trotta et al., 2012). The following studies by Tan et al. (2000) and Myhre et al. (2008) observed a higher frequency of the +3 allele in PD cases compared with healthy controls while in the study by both Tan et al. (2003) and Spadafora et al. (2003) no significant differences of the various genotypes between PD and controls were found in population of Singapore and Italy. However, the study in Italy population, have also shown evidence of association for allele +2 on NACP-Rep1 (Trotta et al., 2012). In 2006, a meta-analysis of 11 study populations provided strong evidence that the 263bp allele was more frequent in PD cases increasing risk of this disease while the 261bp allele did not differ between PD cases and unaffected controls but the authors suggested, that the lack of association of the +2 allele in the meta-analysis could be due to the large fluctuation in its frequencies observed in the analyzed populations (Maraganore et al., 2006). Therefore the aim of the study was analysis of NACP-Rep1 region in PD patients and in controls in Polish population.

2.1.1. Patients

The studies were conducted on 90 patients with PD [SPD patients, 10 with early onset of PD, EOPD, and 80 with late onset of PD, LOPD patients), including 42 women and 47 men aging 34-82 years. Control group included 113 individuals, 79 women and 34 men, 39-83 years of age. Demographic data of all groups summarized in Table 1.

Patients with PD were diagnosed using the criteria of UK Parkinson's Disease Society Brain Bank (Litvan et al., 2003), however stage of disease according to the scale of Hoehn and Yahr (Hoehn & Yahr, 1967).

None of the control subjects had verifiable symptoms of dementia or any other neurological disorders. All subjects had negative family history of PD. All patients were recruited from the Neurology Clinic of Chair and Department of Neurology, University of Medical Sciences, Poznan in Poland. Only Caucasian, Polish subjects were included in this study. A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

Factor	Controls	Patients with PD
Individuals	113	90
Age	39-83	34-82
Mean age ±SD	55.5±9.5	61.9±10.1
F/M	79/34	42/47

Table 1. Demographic data of patients with PD and control subjects analyzed for NACP-Rep1 region in *SNCA* promoter. SD – standard deviation, F – female, M – male.

2.1.2. Genetic investigations

Isolation of DNA. DNA was isolated from peripheral blood lymphocytes by fivefold centrifugation in a lytic buffer, containing 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4, in the presence of buffer containing 75 mM NaCl, 9 mM Na_2EDTA, pH 8.0, and sodium dodecyl sulfate and proteinase K (Sigma, St. Louis, MO). Subsequently, NaCl was added, the lysate was centrifuged, and DNA present in the upper layer was precipitated with 98% ethanol. Extracted genomic DNA was stored at -80°C.

Analysis of G88C mutation of SNCA gene. For exon 3 of *SNCA* analysis, a total of 20 ng gDNA was amplified in 25 μl PCR reactions using specific primers (5'-AAGTGTATTT-TATGTTTTCC-3'; 5'-AACTGACATTTGGGGTTTACC-3') [Lin et al., 1999] and empirically defined reaction conditions. The PCR product was digested with MvaI (Fermentas, Canada) according to Kruger et al. (1998) method for screening c.88 G>C mutation in *SNCA* gene.

Analysis of NACP-Rep1 polymorphism of SNCA promoter region. For analysis of NACP-Rep1 region of *SNCA* promoter, analyzed region was amplified using described previously primers

http://dx.doi.org/10.5772/57107

(5′-GACTGGCCCAAGATTAACCA-3′; 5′- CCTGGCATATTTGATTGCAA-3′) under the conditions: (95°C 30′′, 64°C 45′′, 72°C 30′′) [Tan et al., 2003]. One of the primers was labeled with fluorescent marker – FAM. Sizing of the PCR products was performed by capillary electrophoresis on the 3130xl Genetic Analyzer (Applied Biosystems HITACHI, USA) using GeneScan Size Standard 600LIZ (Applied Biosystems, USA) and controls. The results of electrophoresis were analyzed using Peak Scanner Software v.1.0 (Applied Biosystems, USA). Genotypes were differentiated according to the length of the PCR product. Designations of alleles was followed those previously described (Farrer et al., 2001; Xia et al., 2001).

Moreover, random duplicate samples (10%) were genotyped for all assays for quality control with 100% reproducibility.

Statistical analysis. Statistical analysis was performed using Statistica for Windows Software. The level of significance was set at 5%. Chi-square test and Fisher's exact probability test, test for the two components of the structure, univariate odds ratio (ORs) and logistic regression analysis were used to compare the categorical variables and distribution of alleles and genotypes. The allele frequencies of PD patients and controls were evaluated with regards to Hardy-Weinburg equilibrium using standardized formula.

2.1.3. Results

Screening for mutation c.88 G>C of *SNCA* gene in patients with PD and neurologically healthy controls detected no mutations in both group allow the exclusion of FPD determined by this mutation.

Using PCR amplification and capillary electrophoresis five previously described polymorphic alleles of NACP-Rep1 region in *SNCA* promoter were identified (designated -1, 0, +1, +2, +3) [Farrer et al., 2001; Xia et al., 2001]. Alleles and genotypes frequencies were in Hardy-Weinburg equilibrium in both groups: PD and controls with the exception of alleles +1 and +2, which frequencies in PD patients differed significantly from the expected frequencies calculated from Hardy-Weinburg equilibrium (exact test; $p=0.032$ and $p=0.006$ respectively). The frequency of allele +1 (Table 2) was significantly higher in healthy controls as compared to PD patients ($p<0.001$). In contrast to the allele +1, the frequency of alleles +2 and +3 were significantly higher in PD patients as compared to controls ($p<0.01$; $p<0.05$ respectively). However, the frequency of allele 0 was similar between PD and controls. Moreover, presence of allele -1 was detected only in control subjects (Polrolniczak et al., 2012).

The frequency of +1/+1 genotype was almost fourfold higher in control group than in PD patients ($p<0.001$) whereas the frequency of the genotype +1/+2 was similar in both groups (Table 3). Comparisons of +2/+2 genotype frequencies between PD patients and control group revealed no significant differences but the frequency of this genotype was almost twofold higher in PD patients as compared to controls ($p=0.056$). It has been also detected, that the frequency of +2/+3 was significantly higher in PD patients compared to controls and was almost threefold higher in PD patients ($p<0.05$). Moreover, genotype +1/+3 has been detected only in one PD patient while genotype -1/+1 occurred only in controls (Table 3).

Allele	Controls	Patients with PD
-1	1%	0%
0	5%	6%
+1	53%	33%***
+2	40%	54%**
+3	2%	7%*
Total subjects number	226	180

Table 2. NACP-Rep1 alleles frequency in PD patients and controls. Results are expressed as a percentage. Test for two components of the structure was used. Differences significant at: *p<0.05; **p<0.01; ***p<0.001, as compared to the controls.

Genotypes	Controls	Patients with PD
-1/+1	2%	0%
0/+1	7%	7%
0/+2	3%	4%
+1/+1	23%	6%***
+1/+2	50%	47%
+1/+3	0%	1%
+2/+2	12%	22%
+2/+3	4%	13%*
Total subjects number	113	90

Table 3. NACP-Rep1 genotype frequencies in PD patients and in controls. Results are expressed as a percentage. Test for two components of the structure was used. Differences significant at: *p<0.05; ***p<0.001, as compared to the controls.

Logistic regression analysis have shown, that PD risk (as measured by OR, Table 4) has been reduced in presence of allele +1 and reduces with increasing dose of +1 allele. Moreover, OR pointed to the association the presence of allele +2 with increased risk of PD manifestation in dose dependent manner. Influence of the presence of allele +3 of the increase PD risk has been detected only in heterozygous variant. Genotype +3/+3 have not been detected in any person in both control and PD patient group.

http://dx.doi.org/10.5772/57107

Allele	Heterozygous model		Homozygous model		Common odds ratio	
OR (95% CI)	p	OR (95% CI)	p	OR	p	
-1	-	>0,05	-	>0.05	-	>0.05 (F)
0	-	>0,05	-	>0.05	-	>0.05 (C)
+1	0.406 (0.210-0.785)**	**<0.01**	0.107 (0.035-0.322)***	**<0.001**	0.342***	**<0.001 (C)**
+2	2.719 (1.292-5.719)**	**<0.01**	4.615 (1.774-12.009)**	**<0.001**	2.163***	**<0.001 (C)**
+3	4.601 (1.445-14.647)**	**<0.01**	-	>0.05	4.601**	**<0.01 (F)**

Table 4. Modulation of PD risk manifestation by NACP-Rep1 variants measured by odds ratio. Logistic regression analysis, Fisher's exact test and Chi square test were used. OR – odds ratio; CI – confidence interval; F-Fisher's exact test; C-Chi square test. Differences significant at: **p<0.01; ***p<0.001, as compared to the controls.

Our results similarly to studies in the European, Australian and American populations indicated that, the presence of genotype +1/+1 may reduce PD risk while another study failed to replicate the finding in population of Italy (Fuchs et al., 2008; Maraganore et al., 2006; Mellick et al., 2005; Polrolniczak et al., 2012; Spadafora et al., 2003; Tan et al., 2003; Trotta et al., 2012). It is suggested, that reduction of PD risk by genotype +1/+1 may be related with decreasing ASN expression (Chiba-Falek et al., 2006; Fuchs et al., 2008). In the study in Polish population it has been also observed, in PD patient with genotype +1/+1 tendency to slower progression of the disease and better response to pharmacotherapy at using low doses of the L-dopa treatment compared the other genotypes of NACP-Rep1 (Polrolniczak et al., 2012). It seems, that in PD patients with genotype +1/+1 reduced ASN level, due to reduce ASN aggregation and maintenance of dopamine homeostasis in the central nervous system (CNS) probably leads to milder course of disease compared to patients with other genotypes of NACP-Rep1 (Maguire-Zeiss et al., 2005).

Although the study in Singapore and Italian populations shown no association for alleles +2 and +3 with PD our results confirming the study in populations: German, Italian, Japanese, and multipopulation research detected higher frequency of those alleles in PD patients compared with controls and indicated association of genotypes +2/+2 and +2/+3 with increased risk of PD in Polish population (Maraganore et al., 2006; Mellick et al., 2005; Polrolniczak et al., 2012; Spadafora et al., 2003; Tan et al., 2003; Trotta et al., 2012). It is believed that the influence of genotype +2/+2 and +2/+3 on the risk of PD most likely may be associated with over-expression of ASN, leading to increased aggregation of ASN and the severity of the neurotoxic effect (Chiba-Falek et al., 2006; Cronin et al., 2009; Fuchs et al., 2008). Furthermore in our study in patients with genotypes +2/+2 and +2/+3 we observed tendency to faster progression of the disease but no association with response to therapy (Polrolniczak et al., 2012). This observations seems corresponding with the results of the study by Ritz et al. (2012) shoved that risk of faster decline of motor function was increased four-fold in carriers of the +3 allele of NACP-Rep1 promoter variant. Moreover, the study by Kay et al. (2008) have

indicated a trend of decreasing onset age with increasing allele size while the other study have shown, that age at onset of carriers of at least one allele +2 was earlier compared to noncarriers (Hadjigeorgiou et al., 2006).

However, in contrast to the results of Kay et al. (2008) in Polish population it has not indicated any association of allele 0 with risk of PD, however presence of this genetic variant was correlated in Spearman correlation test (p=0,019; r=-0,507) with decrease in stage of disease in patients suffering for PD over 10 years compared patients with the other genotypes of NACP-Rep1 (Polrolniczak et al., 2012).

It seems that examination of genotypes of region NACP-Rep1 of *SNCA* promoter may help to explain the pathogenesis of PD, as well as facilitate early diagnosis and determine the degree of risk for this neurodegenerative disease.

2.2. Mutations in *PRKN* in the patients with Parkinson's disease

Mutations of *PRKN* were first identified in Japanese families with autosomal recessive juvenile Parkinsonism and since then more than 100 mutations in this gene have been found. Mutation in *PRKN* gene encoding Parkin, have been found both in the EOPD (<40 years) and in the LOPD (>40 years) forms of PD (Bardien et al., 2009; Kitada et al., 1998). Although *PRKN* mutations have been identified in all 12 exons of this gene, the most common seem to be mutations in exons 2, 4, 7, 8, 10 and 11. The vast majority, FPD conditioned by *PRKN* mutation is inherited as an autosomal recessive but it has been also reported heterozygous mutations related with PD manifestation.

Furthermore, it has been shown that mutations in the gene *PRKN* occur at different frequencies both in Caucasians and in populations of African and Asian countries (Kitada et al., 1998; Lucking & Briece, 2000). However, the literature on the prevalence of mutations in *PRKN* and their involvement in the modulation of PD risk are very diverse and have a wide variation depending on the studied population, and the age of subjects included in the study.

It is suggested, that mutations in *PRKN*, including homo- and heterozygous mutations are detected in about 40-50% of early-onset FPD and in about 1.3-20% of SPD patients (Choi et al., 2008; Herdich et al., 2004; Kann et al., 2002; Mellick et al., 2009; Sironi et al., 2008).

The study by Abbas et al. (1999), point mutations of *PRKN* in the European population were approximately twice as common as homozygous exonic deletions. In the European population it has been reported *PRKN* mutations in about 19% of SPD and 50% of early-onset FPD (Lucking & Briece, 2000). Further the study by Lucking et al. (2001) in the sporadic cases revealed that 77% with age of disease onset below 20 years had mutations in *PRKN* gene, but in cases with age of disease onset between 31 and 45 years mutations were found only in 3% in European population. The larger cases studies have confirmed reports of Lucking et al. (2001) and it has shown *PRKN* mutations in 67% of cases with age of onset below 20 years and in 8% of cases with an age of onset between 30–45 years. In another study involving 363 affected subjects from 307 families it has identified *PRKN* mutations in 2% of all late-onset families screened, thereby directly implicating the *PRKN* gene in LOPD (Oliveira et al., 2003). In population of Korea it has been detected *PRKN* mutations in EOPD in 5% frequency while in Japanse

population in 11% (Hattori et al., 1998). In Italian population mutations of *PRKN* occurred in frequency 8-13%, in French in 16%, in German in 9% and in Americans in 4% while in North African in 21%, and in Brazilian in about 8% (Chen et al., 2003; Klein et al., 2005; Lucking & Briece, 2000; Periquet et al., 2003).

The observation, that mutations in the *PRKN* gene are common in juvenile- (JPD) and EOPD and increasing evidence supporting a direct role for Parkin in LOPD make this gene a particularly compelling candidate for intensified investigation. However, despite previous reports, significance of *PRKN* mutation and polymorphism in pathogenesis of PD is still not clear.

The aim of the study was to estimate the frequency of *PRKN* mutation in Polish PD patients and controls.

2.2.1. Patients

According to the inclusion and exclusion criteria a total of 199 subjects were included in this study: 87 SPD patients (10 EOPD patients, and 77 sporadic LOPD patients), including 41 women and 45 men aging 34-82 years. Control group included 112 individuals, 78 women and 34 men, 39-83 years of age. Demographic data of all groups summarized in Table 5. Patients with PD were diagnosed using the criteria of UK Parkinson's Disease Society Brain Bank (Litvan et al., 2003), however stage of disease according to the scale of Hoehn and Yahr (Hoehn & Yahr, 1967). None of the control subjects had verifiable symptoms of dementia or any other neurological disorders. All subjects had negative family history of PD. All patients were recruited from the Neurology Clinic of Chair and Department of Neurology, University of Medical Sciences, Poznan in Poland. Only Caucasian, Polish subjects were included in the study. A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

Factor	Controls	Patients with PD
Individuals	112	87
Age	39-83	34-82
Mean age ±SD	55.6±9.5	61.4±9.9
F/M	78/34	41/45

Table 5. Demographic data of patients with PD and control subjects analyzed for *PRKN* mutation. SD – standard deviation, F – female, M – male.

2.2.2. Genetic investigations

Isolation of DNA. See point 2.1.2

Analysis of deletion of exon 2 and 4 of PRKN gene. Exon deletion in *PRKN* was examined by amplifying exon 2 and 4 using internal and external specific primers previously described (5'-ATGTTGCTATCACCATTTAAG-3'; 5'-AGATTGGCAGCGCAGGCGGCA-3' for exon 2)

[Choi et al., 2008] or generated using the online software Pimer3 (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) based on the published genomic sequence of the *PRKN* gene (5'-TTTCCCAAATATTGCTCTA-3'; 5'-GCAGTGTGGAGTAAAGTTCAAGG-3' for exon 2 and 5'-GCATTATTAGCCACTTCTTCTGC-3'; 5'-TGCTGACACTGCATTTCCTT-3'; 5'-AGATTTCACTCTTGGAGCATAAA-3'; 5'-CAAAGGCGCATAAACGAAA-3' for exon 4). PCR cycling conditions were empirically defined (Polrolniczak et al., 2012).

Analysis of exon 4, 7 and 11 of PRKN gene. High resolution melting (HRM) were used for mutation screening in exon 4, 7 and 11 of *PRKN*. HRM was performed with the LightCycler 480 Real-Time PCR system (Roche, USA) and High Resolution Master Mix (Roche, USA). Reactions were performed on 96-well plates, using 5 ng of template DNA, 1x Master Mix, 2.5 mM MgCl2, and 10 pmol primers (on request) in a 10 μl reaction volume. PCR cycling conditions comprised of an initial denaturation step of 95°C for 5 min, 30 cycles of denaturation at 95°C for 15 s, annealing at 64°C (or 63°C for exon 4) for 15 s, extension at 72°C C for 15 s, and a final extension step of 72°C for 7 min. HRM analysis was performed from 55°C to 95°C (Polrolniczak et al., 2012). Melting curves and difference plots were analyzed by 3 investigators blinded to phenotype. For the samples with shifted melting curves, PCR products were cleaned and sequenced in the forward and reverse directions. Sequencing was performed using the 3130xl Genetic Analyzer (Applied Biosystems HITACHI, USA) and reads were aligned to the human reference genome with BioEdit Software (Tom Hall Ibis Biosciences, Canada). Coding DNA mutation numbering is relative to NM_004562.2.

Analysis of c.930 G>C substitution in exon 8 of PRKN gene. For exon 8 of *PRKN* analysis 20 ng gDNA was amplified in 25 μl using PCR reaction with specific primers (5'- CTAAA-GAGGTGCGGTTGGAG-3'; 5'- GGAGCCCAAACTGTCTCATT-3') generated using the online software Pimer3 based on the published genomic sequence of the *PRKN* gene. PCR cycling conditions were empirically defined. Screening for the c.930 G>C mutation of *PRKN* was performed by a RFLP analysis on 2% agarose gels using Mva I (Fermentas, Canada) as restriction enzyme. All detected mutations were confirmed by sequencing of PCR product.

Moreover, random duplicate samples (10%) were genotyped for all assays for quality control with 100% reproducibility.

Statistical analysis. Statistical analyses were performed using Statistica for Windows Software. The level of significance was set at 5%. Chi-square test and Fisher's exact probability test, univariate odds ratio (ORs) and logistic regression analysis were used to compare the categorical variables and distribution of alleles. The allele frequencies of PD patients and controls were evaluated with regards to Hardy-Weinburg equilibrium using standardized formula.

2.2.3. Results

Analysis of deletions of exons 2 and 4 *PRKN* has detected no genetic changes both in PD patients and control group. However, point mutation screening in patients with PD and healthy controls identified 5 missence substitutions which were almost fourfold more frequent in PD patients as compared with controls ($p<0.001$) [Table 6]. We also showed, that the presence

http://dx.doi.org/10.5772/57107

of *PRKN* substitution increased risk of PD over six-fold (p<0.001; OR=6.059). All substitutions were non-synonymous and were in heterozygous state.

	Controls	Patients with PD	OR	95% CI	p
PRKN mutations	8%	31%***	6.059	2.188-11.207	<0.001 (C)
Total subjects number	112	87	-	-	-

Table 6. Total *PRKN* point mutations frequencies in PD patients and controls. Results are expressed as a percentage. Chi square test was used. OR – odds ratio; CI – confidence interval; C - Chi square test. Differences significant at: ***p<0.001, as compared to the controls.

In exon 4 of *PRKN* two mutations were detected: c.500 G>A transition leads to S167A substitution (with frequency sevenfold higher in PD than in control group; p<0.05) and a novel heterozygous mutation c.520 C>T resulting L174F substitution and occurring only in PD patients. Furthermore, first time in Polish population we detected c.823 C>T (exon 7, R275T; only in PD) and c.930 G>C (exon 8, E310D) substitutions (over threefold more frequently in PD than in controls; p<0.01). Moreover, we detected also a transition c.1180 G>A in exon 11 of *PRKN*. It has been also shown, that c.500 G>A, c.930 G>C and c.1180 G>A substitutions significantly increased PD risk (Table 7). Simultaneously analysis of the amino acid sequence of the Parkin (encoded by *PRKN* gene) revealed that the substitution E310D and L174F are located in conserved region whereas substitution R275T and D394N in a limited conserved region of this protein (Polrolniczak et al., 2012).

Mutation/ polymorphism	c.500 G>A	c.520 C>T	c.823 C>T	c.930 G>C	c.1180 G>A
Controls	1%	0%	0%	5%	2%
PD patients	7%*	2%	1%	18%**	11%**
OR	8.000	-	-	3.926	6.938
95% CI	0.945-67.712	-	-	1.436-10.735	1.480-32.528
p	<0.05 (F)	>0.05 (F)	>0.05 (F)	<0.01 (C)	<0.01 (C)

Table 7. *PRKN* point mutations frequencies in PD patients and controls. Results are expressed as a percentage. Logistic regression analysis, Fisher's exact test and Chi square test were used. OR – odds ratio; CI – confidence interval; F - Fisher's exact test; C - Chi square test. Differences significant at: *p<0.05; **p<0.01 as compared to the controls.

Additionally in 5% PD patients it has been detected more than one mutation in *PRKN* gene while all control subjects who had substitution in *PRKN*, had only one mutation (Table 8).

Coexistence of substitutions in *PRKN* gene	Percentage of PD patients
c.823 C>T , c.1180 G>A	1%
c.500 G>A, c.520 C>T	1%
c.930 G>C, c.1180 G>A	2%
c.500 G>A, c.930 G>C, c.1180 G>A	1%

Table 8. Coexistence more than one *PRKN* point mutations in PD patients in Polish population (Polrolniczak et al., 2012).

It is suggested, that single or multiple exon deletions and duplications occur with a frequency of 15.8% and account for about 50% of all mutations of *PRKN* gene (Nuytemans et al., 2010). Nevertheless, althought many reports indicated important role of *PRKN* exons 2 and 4 deletions in pathogenesis of idiopatic PD (Choi et al., 2008; Guo et al., 2010; Macedo et al., 2009; Pankratz et al., 2009) in the study in Polish population it has not detected any deletion of exon 2 and 4 in *PRKN* gene as opposed to the German and Japan population, as well as the results obtained in the multipopulation study (Cookson et al., 2008; Nishioka et al., 2009; Polrolniczak et al., 2011; 2012; Shapira et al., 2002). On the other hand, our results were consistent with the study by Kruger et al. (1999), Sinha et al. (2005), as well Barsottini et al. (2011). However, it not be ruled out, that Polish patients have deletion of other not tested exons. Oliveri et al. (2001) suggested that deletion mutations of *PRKN* were not as common in LOPD as in EOPD. Therefore, it seems that copy number variation of *PRKN* is most probably related with EOPD (Wang et al., 2004).

However, point mutations in *PRKN* gene although they are characteristic for EOPD, currently it is suggested that it can be also involved in the pathogenesis of LOPD. However, studies utilizing common mutations and polymorphisms in tests for association with LOPD have produced mixed results (Hu et al., 2000; Oliveri et al., 2001; Satoh & Kuroda, 1999; Wang et al., 1999).

Furthermore there is no question that Parkin-associated parkinsonism is recessive; that is, both alleles are mutant, but despite previous reports whether a heterozygous mutation can cause or increase the risk for PD remains an issue of debate (Farrer et al., 2001; Klein et al., 2000; Lucking et al., 2001; Maruyama et al., 2000).

In the German population the frequency of *PRKN* mutations was 9% (Kann et al., 2002), in Brazilian population 8% (Periquet et al., 2001), and in the American population reached value of less than 4% (Chen et al., 2003) while in the Japanese population reached 66% (Hattori et al., 1998). In Polish population it has been showed small share of *PRKN* mutation in the pathogenesis of EOPD (Dawson & Dawson, 2003) while our study in LOPD have shown, that *PRKN* mutation in Polish population occurred with frequency 20,6% (Polrolniczak et al., 2011; 2012) what was similar to SPD in European population (Nishioka et al., 2009).

Moreover, we showed, that in the Polish population the most frequently were polymorphisms c.500 G>A, c.1180 G>A and c.930 G>C of *PRKN*. Simultaneously, it appears that these polymorphisms may have incomplete penetration or lead to preclinical changes in the CNS and

http://dx.doi.org/10.5772/57107

increased risk LOPD probably in combination with other genetic or environmental factors, as evidenced by Bardien et al. reports (2009). The other two identified *PRKN* mutations (c.823 C> T, c.520 C> T) were detected only in PD patients, what may indicate a high penetration of these substitutions (Sinha et al., 2005), while novel mutation c.520 C>T was identified in two patients and led to a relatively early onset of disease before age 40.

It is suggested, that haploinsufficiency may be considered as a reduction of normal gene expression accompanied by a loss of normal protein activity. Moreover, a lot of reports indicate to the existence of a second, undetected mutation in these patients, perhaps in the promoter or intronic regions (Giasson & Lee, 2001).

Our results, also suggests that the presence more than one heterozygous mutation in the *PRKN* gene may be necessary to PD manifestation. This hypothesis was first proposed by Abbas et al. (1999) moreover, later reviews generally assume the existence of a second, undetected mutation (Giasson & Lee, 2001). In our study also it is probably that patient who had one mutation in *PRKN* may have more genetic changes in not tested region of the gene so extension the studies of the other region of *PRKN* gene is necessary to clarify this issue. On the other hand it can not be ruled that one heterozygous mutation in *PRKN* may be sufficient to increase risk of PD and induce preclinical changes in *substantia nigra* (Khan et al., 2005).

Finally, it seems that clinically, PD patients with *PRKN* substitution generally are characterized by slower progression of the disease compared with PD patients without mutation. Moreover, it has been also observed, that in PD patients with *PRKN* mutations response to L-dopa therapy has been better than in PD patients without substitutions. This observation are generally consistent with the typical descriptions of *PRKN* patients which present slow disease progression (Abbas et al., 1999; Lucking & Briece, 2000) and good response to L-dopa treatment although it have been showed that patients with *PRKN* mutation were more likely to develop treatment-induced motor complications earlier in the treatment (Khan et al., 2005; Lucking & Briece, 2000).

It seems, that point mutation in *PRKN* gene may be involved in the pathogenesis of LOPD and modulate clinical futures in this disease. It is also probably, that analysis of mutations in *PRKN* gene may be useful for diagnostic and prognostic process in PD.

2.3. Mutations in *HTRA2* and *SPR* in the patients with Parkinson's disease

It seems that presence of mutation in the other genes involved in the pathogenesis of PD like *SPR* (involved in dopamine biosynthesis) and *HTRA2* (involved with mitochondrial pathway of PD) probably may additionally affect the levels of ASN and Parkin through interaction with these proteins (Bogaerts et al., 2008; Karamohamed et al., 2003; Sharma et al., 2006; Sharma et al., 2011; Strauss et al., 2005). However, role of those genes in pathogenesis of PD is not enough known. The serine protease HTRA2 is localized to the inner membrane space of mitochondria (Suzuki et al., 2001). Mitochondrial dysfunction as well as ubiquitin–proteasome system damage has been proposed as possible mechanisms leading to dopaminergic neuronal degeneration (Lin & Beal, 2006; Malkus et al., 2009; Rubinsztein, 2006). Therefore *HTRA2*

likewise *PRKN* may be included in mitochondrial pathway of PD independently of *PRKN* but that does not exclude the effects of dysfunction of *HTRA2* and *PRKN* may be additive.

Locus of *HTRA2* gene was recently assigned the PARK13 name, but the association of HTRA2 mutations and PD has not been confirmed in independent studies yet. In the study by Strauss et al. (2005) a single heterozygous *HTRA2* substitution (c.1195 G>A, Gly399Ser) was detected in four German patients with sporadic PD while another substitution (c.421 G>T, Ala141Ser) was more frequently found in PD than in controls. However, in a recent study by Simin-Sanchez & Singleton (2008) both variations were not associated with PD while in Belgian population it have been detected another substitution of *HTRA2* (c.1210 C>T, R404W) in sporadic PD (Bogaerts et al., 2008). These inconsistent findings raise a question about the role of mitochondrial *HTRA2* in PD susceptibility.

SPR gene is located in region covered by the locus PARK3 on chromosome 2p13 (Gasser et al., 1998), but the gene responsible for PD in PARK3 families has not yet been identified. One of the candidates is *SPR* gene. The study of Karamohamed et al. (2003) refined association to a region containing the *SPR* gene with PD, and it have been confirmed in further reports, but the study by Sharma et al. (2011) have not shown the association *SPR* and PD risk (Karamohamed et al., 2003; Sharma et al., 2006; 2011). However, authors emphasize varied genetic distributions between different populations (Sharma et al., 2011). It is known, that SPR is involved in dopamine synthesis and likewise ASN probably may be responsible for disturbances in methabolisme of dopamine. The study of Tobin et al. (2007) has shown that expression of *SPR* was significantly increased in PD patient compared with controls. However mutations in *SPR* in PD have not been analyzed so far. Moreover, it is known that phosphorylation of *SPR* increase sensitivity for protease activity and that in human SPR protein phosphorylated is only Ser213 (Fujimoto et al., 2002). Therefore, we decided to search for mutation in codon 213 of *SPR* in PD cases.

2.3.1. Patients

The studies were conducted on 89 patients with PD (10 EOPD patients, and 79 sporadic LOPD patients), including 41 women and 47 men aging 34-82 years. Control group included 113 individuals, 79 women and 34 men, 39-83 years of age. Demographic data of all groups summarized in Table 9.

Patients with PD were diagnosed using the criteria of UK Parkinson's Disease Society Brain Bank (Litvan et al., 2003), however stage of disease according to the scale of Hoehn and Yahr (Hoehn & Yahr, 1967).

None of the control subjects had verifiable symptoms of dementia or any other neurological disorders. All subjects had negative family history of PD. All patients were recruited from the Neurology Clinic of Chair and Department of Neurology, University of Medical Sciences, Poznan in Poland. Only Caucasian, Polish subjects were included in the study. A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

http://dx.doi.org/10.5772/57107

Factor	Controls	Patients with PD
Individuals	113	89
Age	39-83	34-82
Mean age ±SD	55.5±9.5	62.0±10.1
F/M	79/34	41/47

Table 9. Demographic data of patients with PD and control subjects analyzed for *HTRA2* and *SPR* mutations. SD – standard deviation, F – female, M – male.

2.3.2. Genetic investigations

Isolation of DNA. See point 2.1.2

Analysis of HTRA2 gene. Exons 1 and 7 of *HTRA2* were amplified in 25 μl by PCR under the empirically defined conditions. Primers to amplify (on request) were generated using the online software Pimer3 based on the published genomic sequence of the *HTRA2* gene. PCR products were digested with MboII, MvaI and MspI restriction enzymes (Fermentas, Canada) for screening for c.421 G>T, c.1195 G>A and c.1210 C>T mutations (respectively) and analyzed on 2.5% agarose gels.

Analysis of SPR gene. Exon 3 of *SPR* gene were amplified using PCR with specific primers (5'-TCCATGTTCAGTGGGCTTTT-3'; 5'- TTTCTGGGCTGACACCTTG-3') generated with Primer3 software under the empirically defined conditions. Screening for the c.637 T>A and c.637 T>G mutations of *SPR* was performed by a RFLP analysis on 2.5% agarose gels using TaaI and SsiI (Fermentas, Canada) as restriction enzymes. All detected mutations were confirmed by sequencing of PCR product. Moreover, random duplicate samples (10%) were genotyped for all assays for quality control with 100% reproducibility.

Statistical analysis. See point 2.2.2.

2.3.3. Results

In Polish population the presence of *HTRA2* point mutation was detected in 3% of PD patients (in 2% - c.1195 G>A resulting A141S substitution and in 1% c.421 G>T leads to G399S substitution) and none of controls (Table 10). However, c.1210 C>T mutation of *HTRA2* has not occurred both in PD patients and controls.

Mutation/polymorphism	c.421 G>T	c.1195 G>A	c.1210 C>T	Total substitutions
Controls	0%	0%	0%	0%
PD patients	1%	2%	0%	3%
OR	-	-	-	9.080
95% CI	-	-	-	-
p	>0.05 (F)	>0.05 (F)	>0.05 (F)	=0.05 (F)

Table 10. *HTRA2* point mutations frequencies in PD patients and in controls. Results are expressed as a percentage. Logistic regression analysis and Fisher's exact test were used. OR – odds ratio; CI – confidence interval; F-Fisher's exact test.

In 213 codon of *SPR* gene novel mutation c.637 T>A was identified in 4% patients with PD and 2% controls (Table 11). This substitution is non-synonymous and leads to S213T changes in amino acid chain. However, we did not detected the second analyzed substitution c.637 C>G *SPR* in any of the subjects.

Mutation/polymorphism	c.637 T>A	c.637 C>G
Controls	2%	0%
PD patients	4%	0%
OR	-	-
95% CI	-	-
p	>0.05 (F)	>0.05 (F)

Table 11. *SPR* point mutations frequencies in PD patients and in controls. Results are expressed as a percentage. Logistic regression analysis and Fisher's exact test were used. OR – odds ratio; CI – confidence interval; F-Fisher's exact test.

In PD patients with substitutions in *HTRA2* gene it have been observed slower progression of the disease wherein there was statistically significant association (Spearman correlation test) transition c.1195 G>A with decrease stage of disease ($p=0.029$; $r=-0.237$) but association of c.421 G>T substitution have not been significant and have remained at the level of trend. Furthermore it have been shown, that using in PD patients with *HTRA2* mutations doses of L-dopa were lower than in patients without mutations and the response to therapy was better in presence of substitution. Finally, it seems that identified *HTRA2* mutations may be one of PD risk factor, especially since Strauss et al. (2005) demonstrated the presence of olfactory dysfunction in asymptomatic *HTRA2* mutation carrier.

Moreover, it seems that mutation c.637 T>A, because of localization, probably may affect phosphorylation of SR and thereby its activity and finally regulate biosynthesis of DA and serotonin (5-HT). However, analysis of expression and functional testing are necessary to explain importance and role of this mutation. Nevertheless, what is important, in our study c. 637 T>A *SPR* mutation has been significantly associated in Spearman correlation test, with the presence of depressive symptoms in PD patients ($p<0.0001$; $r=0.371$) probably by regulating the level of 5-HT (McHugh et al., 2009). Simultaneously, the presence of c.637 T>A of *SPR* mutation in PD patients have not been associated with differences in progression of the disease, response to L-dopa therapy, amount using L-dopa dose or presence of dementia compared to PD patients without *SPR* mutation.

2.4. Coexistence of mutations in more than one gene (*SNCA*, *PRKN*, *HTRA2* and *SPR)* in the patients with Parkinson's disease

Our study indicated, that in PD patients as well as in controls in the Polish population, *PRKN* mutations most frequently accompanied by the presence of genotype +1/+2. Interesting the coexistence of mutations *PRKN* with genotypes +2/+2 and +2/+3 have been demonstrated only

http://dx.doi.org/10.5772/57107

in patients with PD (Fig. 1). It seems that co-occurrence of point mutation of *PRKN* and polymorphism of *SNCA* promoter region may in additive manner increase risk of PD manifestation.

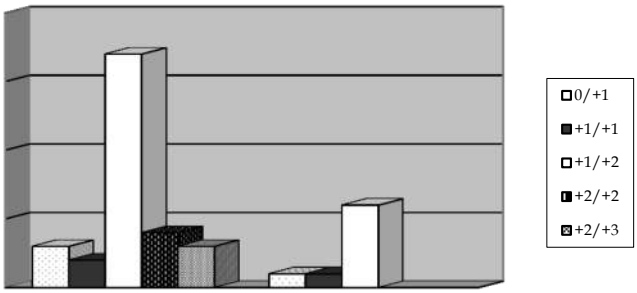

Figure 1. The frequency of NACP-Rep1 region of *SNCA* promoter variants in subjects with *PRKN* mutations (PD patients and controls).

Furthermore, in the patients with PD we demonstrated coexistence of point mutations in *PRKN* and *SPR* or *PRKN* and *HTRA2* genes (Table 12). However, in controls, coexistence of mutations *PRKN* and *SPR* have been observed also in one person. Therefore it seems that in patients with mutation of *PRKN* and *HTRA2* genes, simultaneous incorrect function of two proteins involved in the mitochondria proper functioning (HTRA2 and Parkin) may additionally increase risk of PD manifestation.

Probe number	Group	Substitutions		
		PRKN	***HTRA2***	***SPR***
25	PD patient	c.500 G>A	-	c.637 T>A
114	PD patient	c.930 G>C	c.1195 G>A	-
202	PD patient	c.930 G>C	c.421 G>T	-
18	Control	c.930 G>C	-	c.637 T>A

Table 12. Coexistence of mutations in more than one analyzed gene in PD patients and controls.

3. Role of alpha-synuclein in pathogenesis of Parkinson's disease

Alpha-synuclein is a protein composed of 140 amino acids and is a part of family of proteins with the β- and γ-synuclein (Clayton & George, 1998). For many years, the structure of ASN was determined as the,,not-folded" chain of amino acids, taking the helical form only in conjunction with the lipids of cell membranes. It was thought that the ASN is a monomer form but the recent studies have shown that under physiological conditions ASN largely takes the

form of tetramers, and may take the helical form without connection to the lipid membrane (Bartels et al., 2011).

Immunohistochemical studies have shown that in the cells, there is essentially ASN bonded to both the nuclear membrane, and in the synaptic vesicles (Totterdel & Meredith, 2005). To a lesser extent, ASN occurs in the free form in the cytoplasm.

Functions of ASN are not fully understood, however, due to cellular location of this protein it is suggested, that function of ASN may be related with the synaptic transport (Alim et al., 2002). There are also reports indicating that ASN participate in the process of differentiation and survival of the dopaminergic neuron progenitor cells of the mouse and human (Michell et al., 2007; Schneider et al., 2007).

Under pathological conditions ASN may change the structure and take the form of beta harmonica, what may lead to aggregation of ASN and formation of soluble oligomers, and then the insoluble filaments and deposits in the nerve cells (Bodles et al., 2001). As it have been shown, ASN is one of the main components of Lewy's bodies (LB), pathology, round or polymorphonuclear cellular inclusions in the cytoplasm of nerve cells. Moreover, it is suggested, that the formation of insoluble deposits of ASN and the aggregation process may give rise to the formation of LB (Halliday et al., 2006).

It is obvious that the process of aggregation of the ASN is a negative phenomenon for neural cells not only because of the high toxicity of the resulting aggregates, but also because of the ASN physiological function disorders caused by the reduction of bioavailability of this protein (Conway et al., 2000). It has been shown, that in PD, the process of ASN aggregation may be modulated by a number factors (Fig. 2) [Haggerty et al., 2011; Li et al., 2008; Ren et al., 2009; Sherer et al., 2002].

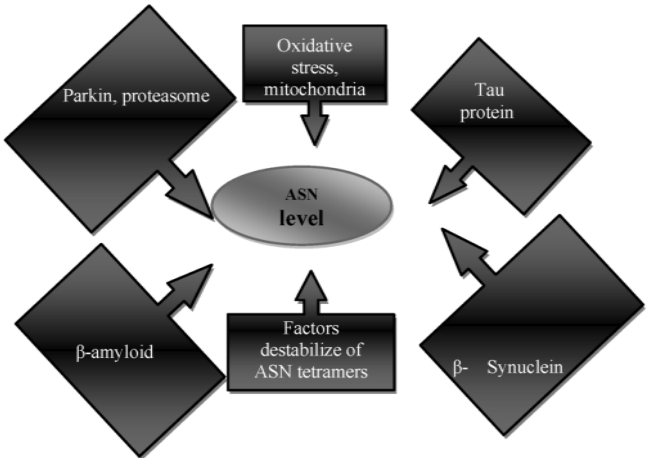

Figure 2. Selected factors affect alpha-synuclein (ASN) level.

http://dx.doi.org/10.5772/57107

3.1. Alpha-synuclein concentration in Parkinson's disease

It has been shown that aggregation of the ASN may be caused among others by multiplication of *SNCA* gene. Furthermore, it has been shown that triplication of *SNCA* gene leads to two-fold increase of ASN level, while duplication of *SNCA* gene increases the level of this protein one and a half-fold (Farrer et al., 2001; Singleton et al., 2003). Therefore it is believed that increased level of ASN may be related with PD manifestation (Farrer et al., 2001; Mata et al., 2004). It is also known that over-expression of ASN in neuron facilitates aggregation of this protein even in the presence of the correct structure of ASN. Moreover, elevated expression of *SNCA*-mRNA levels have been found in the affected regions of PD brain (Chiba-Falek et al., 2006). Increased of ASN level has been also associated with progress and worsening of the disease symptoms (Singleton et al., 2003). However, there are only few reports investigating the level of ASN in the blood of PD patients (Bialek et al., 2011; Fuchs et al., 2008; Lee et al., 2006).

The aim of the study was to estimate the concentration of ASN in plasma of patients with PD and in control group.

3.1.1. Patients

The studies were conducted on 32 patients with PD, including 18 women and 14 men aging 35-82 years. Control group included 24 individuals, 20 women and 4 men, 40-69 years of age. Demographic data of all groups summarized in Table 13.

Patients with PD were diagnosed using the criteria of UK Parkinson's Disease Society Brain Bank (Litvan et al., 2003), however stage of disease according to the scale of Hoehn and Yahr (Hoehn & Yahr, 1967). None of the control subjects had verifiable symptoms of dementia or any other neurological disorders.

All patients were recruited from the Neurology Clinic of Chair and Department of Neurology, University of Medical Sciences, Poznan in Poland. Only Caucasian, Polish subjects were included in the study.

A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

Factor	Controls	Patients with PD
Individuals	24	32
Age	40-69	35-82
Mean age ±SD	55.3±6.8	62.5±10.5
F/M	20/4	18/14

Table 13. Demographic data of patients with PD and control subjects analyzed for ASN concentrations. SD – standard deviation, F – female, M – male.

3.1.2. Analysis of ASN concentrations

Preparation of samples. Blood samples from these subjects were drawn using EDTA as an anticoagulant in the morning after an overnight fast and the samples were centrifuge for 15 min at 1000xg at 4° C within 30 min and plasma was frozen at −80° C for later use.

Determination of ASN concentration. ASN ELISA was performed using the Enzyme-linked Immunosorbent Assay Kit for Human Synuclein Alpha (Uscn Life Science Inc., China) according to the manufacturer's protocol. The minimal detection limits in this assay is typically less than 4.8 pg/ml. The standard curve concentrations used were 1000; 5000; 250; 125; 62.5; 31.2 and 15.6 pg/ml. The intra- and interassay precision of coefficiences of variation were <10% and <12% respectively. After completion of each assay the plate(s) were read at 450 nm on an EPOCH Multi-Volume Spectrophotometer (BioTek, USA) and the results were analyzed using Gen5 2.1 Software (BioTek, USA).

3.1.3. Results

Detectable concentrations of ASN have been detected in higher percentage of controls than in PD patients. However, in patients with PD has been shown higher concentration of ASN (Table 14).

Parameter	Controls	Patients with PD
Detectable concentrations of ASN, n [%]	4 [17%]	4 [12%]
ASN concentrations [pg/ml]	[68.19-645.57]	[55.2-1294.9]

Table 14. ASN concentrations in the patients with PD and in control group. Results are expressed as an interval between the minimum and maximum measurement, n – number, % - of subjects; detectable concentrations of ASN – above 0.058 pg/ml in ELISA test.

In PD patients, the highest concentrations of ASN were present in two first stages of disease progress (Hoehn and Yahr scale) [Table 15] and in the first ten years of the disease (Table 16).

Stage of disease according to the scale of Hoehn and Yahr	ASN concentrations in PD patients [pg/ml]
1	[55.2]
2	[60.94-1294.9]
3	-

Table 15. Detectable (above 0.058 pg/ml in ELISA test) concentrations of ASN in PD patients depending on stage of disease in Hoehn and Yafr scale. Results are expressed as an interval between the minimum and maximum measurement/single result.

http://dx.doi.org/10.5772/57107

Duration of disease	ASN concentrations in PD patients [pg/ml]
< 5 years	[55.2-293.67]
5-10 years	[1294.9]
>10 years	-

Table 16. Detectable (above 0.058 pg/ml in ELISA test) concentrations of ASN in PD patients depending on duration of the disease. Results are expressed as an interval between the minimum and maximum measurement/single result.

In this study and Bialek et al. (2011) have been shown higher concentration of plasma ASN level in PD patients as compared to controls. However, it seems that aggregation of the ASN in the nerve cells may reduce ASN ability to pass through the blood-brain barrier, which in turn may result in significantly reduced levels of this protein in the peripheral blood. Moreover, a high concentration of ASN has been detected only in the initial period of PD (in two first stages of PD progress in Hoehn and Yahr scale, and in the first ten years of the disease), probably even before the accumulation of deposits in the form of LB in the brain of PD patients. However, in the study by Pchelina et al. (2011) the level of ASN was significantly lower in patients with *LRRK2*-associated PD compared with SPD and controls what may be caused also by severed ASN aggregation in this group.

4. Role of Parkin in pathogenesis of Parkinson's disease

Parkin is a cytoplasmic protein which plays a vital role in the proper functioning of the mitochondria and functions as an E3 ligase ubiquitin stimulating protein binding (directed to degradation in the proteasome) with ubiquitin, consequently preventing the cell apoptosis (Zhang et al., 2000). Ubiquitination is a vital cellular quality control mechanism that prevents accumulation of misfolded and damaged proteins in the cell. It is thought that substrates of Parkin include among others synphilin-1, ASN, CDC-rel1, cyclin E, p38 tRNA synthase, Pael-R and synaptotagmin XI. It has been shown in the study by Zhang et al, [2000] Parkin is also responsible for their own ubiquitination and degradation in the proteasome.

Recent studies have shown that Parkin may play a role in decision-making, choosing between two systems of degradation: the proteasome activity (through its ability to promote ubiquitination K48 associated with the proteasome) and macroautophagy (through K63 ubiquitination related to cell signaling and the formation of LB) [Henn et al., 2007; Lim et al., 2006].

4.1. Parkin concentration in Parkinson's disease

The aim of the study was to estimate the concentration of Parkin in plasma of patients with PD and in control group.

Patients (see point 3.1.1.)

Analysis of Parkin concentrations

Preparation of samples. Blood samples from these subjects were drawn using EDTA as an anticoagulant in the morning after an overnight fast and the samples were centrifuge for 20 min at 1000xg at 4° C within 30 min and plasma was frozen at −80° C for later use.

Determination of Parkin concentration. Parkin ELISA was performed using the Enzyme-linked Immunosorbent Assay Kit for Human Parkinson Disease Protein 2 (Uscn Life Science Inc., China) according to the manufacturer's protocol. The minimal detection limits in this assay is typically less than 0.058 ng/ml. The standard curve concentrations used were 10; 5; 2.5; 1.25; 0.625; 0.312; and 0.156 ng/ml. The intra- and interassay precision of coefficiences of variation were <10% and <12% respectively. After completion of each assay the plate(s) were read at 450 nm on an EPOCH Multi-Volume Spectrophotometer (BioTek, USA) and the results were analyzed using Gen5 2.1 Software (BioTek, USA).

4.1.1. Results

Detectable concentrations of Parkin have been detected in similar percentage of controls and PD patients. However, in patients with PD has been shown lower concentration of Parkin (Table 17).

Parameter	Controls	Patients with PD
Detectable concentrations of Parkin, n [%]	5 [21%]	7 [22%]
Parkin concentrations [ng/ml]	[0.036-4.436]	[0.076-2.123]

Table 17. Parkin concentrations in the patients with PD and control group. Results are expressed as an interval between the minimum and maximum measurement. n – number, % - of subjects; detectable concentrations of Parkin – above 0.058 ng/ml in ELISA test.

In PD patients, the highest concentration of Parkin occurred in 2 stage of disease progress with tendency to reduce the concentration in the 3 stage of the disease (Hoehn and Yahr scale) [Table 18] and in the first ten years of the disease (Table 19).

Stage of disease according to the scale of Hoehn and Yahr	Parkin concentrations in PD patients [ng/ml]
1	-
2	[0.158-2.123]
3	[0.076-0.409]

Table 18. Detectable (above 0.058 ng/ml in ELISA test) concentrations of Parkin in PD patients depending on stage of disease in Hoehn and Yahr scale. Results are expressed as an interval between the minimum and maximum measurement/single result.

http://dx.doi.org/10.5772/57107

Duration of disease	Parkin concentrations in PD patients [ng/ml]
< 5 years	[0.549-2.123]
5-10 years	[0.158-2.054]
>10 years	[0.076] mutation in 11 exon of *PRKN*

Table 19. Detectable (above 0.058 ng/ml in ELISA test) concentrations of Parkin in PD patients depending on duration of the disease. Results are expressed as an interval between the minimum and maximum measurement/single result.

It is known that dysfunction of Parkin may lead to manifestation of PD in several mechanism including mitochondrial and ubiquitination disturbances. It also seems that expression and cellular level of Parkin may be essential factor for proper function of this protein. However, the presence of Parkin protein has been demonstrated in human serum using Western blotting, there is few analysis of the level of this protein in the blood of PD patients (Dorszewska et al., 2012; Kasap et al., 2009). In the study by Dorszewska et al. (2012) has been detected lower plasma Parkin concentration in PD patients than in controls. Moreover, increased levels of the Parkin have been detected in PD patients in the early stages of PD (Hoehn and Yahr scale) and decreasing with the progress and duration of this disease. It seems that in the early stages of PD development may occur to increase of Parkin expression through the ongoing degenerative process and to the accumulation of pathological proteins-Parkin substrates. However, as the disease progresses, probably, resources of the Parkin running out and occurs weaken its neuroprotective function.

5. Relationship between alpha-synuclein and Parkin levels in Parkinson's disease

In 2001, Shimura et al. first described the presence in the human brain complex containing Parkin with the glycosylated form of the ASN (alpha-SP22), thus indicating the involvement of Parkin in ASN degradation in ubiquitin-proteasome system [Shimura et al., 2001; Chung et al., 2004]. It has been also shown that dysfunction of the Parkin can lead to ineffective elimination of ASN and the aggregation of this protein [Haass & Kahle, 2001]. In addition, according to the reports, the Parkin may also interact with the dopamine and indirectly influence the aggregation of the ASN in the nerve cell (Oyama et al., 2010). Therefore, it seems that the levels of these two proteins may be related and dependent on each other.

Patients (see point 3.1.1.)

Analysis of ASN (see point 3.1.2.), and Parkin (see point 4.1.2.) concentrations

5.1. Results

In patients with PD detectable levels of Parkin occurred in a nearly two-fold higher incidence than the ASN (Tables 14, 17).

Parameter	Controls	Patients with PD
ASN concentration [pg/ml]	[68.19-645.57]	[55.2-1294.9]
Parkin concentration [pg/ml]	[36.0-4436.0]	[76.0-2123.0]

Table 20. ASN and Parkin concentrations in the patients with PD and control group. Detectable concentrations of ASN and Parkin – above 0.058 pg/ml in ELISA test. Results are expressed as an interval between the minimum and maximum measurement.

Stage of disease according to the scale of Hoehn and Yahr	ASN concentrations in PD patients [pg/ml]	Parkin concentrations in PD patients [pg/ml]
1	[55.2]	-
2	[60.94-1294.9]	[158.0-2123.0]
3	-	[76.0-409.0]

Table 21. Detectable (above 0.058 pg/ml in ELISA test) concentrations of ASN and Parkin in PD patients depending on stage of disease in Hoehn and Yahr scale. Results are expressed as an interval between the minimum and maximum measurement/single result.

Duration of disease	ASN concentrations in PD patients [pg/ml]	Parkin concentrations in PD patients [pg/ml]
< 5 years	[55.2-293.67]	[549.0-2123.0]
5-10 years	[1294.9]	[158.0-2054.0]
>10 years	-	[76.0] mutation in 11 exon of *PRKN*

Table 22. Detectable (above 0.058 pg/ml in ELISA test) concentrations of ASN and Parkin in PD patients depending on duration of the disease. Results are expressed as an interval between the minimum and maximum measurement/ single result.

In this study and studies by Bialek et al. (2011) and Dorszewska et al. (2012) have been shown, that in PD patients increased level of ASN was associated with the decreased level of Parkin in contrast to control group (Tables 20-22). Independently for the analyzed group, the highest levels of ASN have been observed in the subjects who had very low Parkin levels. It suggested that low concentration of Parkin may contribute to increased ASN level in the nerve cells and combined with over-expression of ASN intensify or accelerate neurodegenerative process. Moreover, it has been also shown that configuration: increased plasma level of ASN and decreased of Parkin was associated with earlier onset of this disease.

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6. Mutations in PARK (*PRKN, SPR, HTRA2, SNCA*) genes and ASN and Parkin concentrations in Parkinson's disease

Our study on Polish population shown that in PD patients *PRKN* (exons 4, 8, 11) mutations were more than four times frequency as compared to controls. Moreover, in PD patients more frequently occurred genotypes +2/+2 and +2/+3 of the promoter region *SNCA* gene than in controls. In patients with PD shown higher concentration of ASN while higher Parkin level in controls. In PD patients without mutations in PARK, highest concentration of ASN and Parkin was present in two first stages of disease progress (Hoehn and Yahr scale) and in the first ten years of the disease. However, only in PD patient with mutation in 11 exon of *PRKN* gene shown presence of Parkin without ASN after ten years of disease duration (Table 19, 22).

It seems that analysis of these pathological proteins with PARK gene mutations may be useful in the diagnostic and monitoring of the PD progress in the future.

7. Conclusion

In Polish population, in *PRKN* gene point mutations occur few times more often in PD patients than controls. Control subjects tend to show higher level of plasma Parkin whereas patients suffering from PD tend to generate higher level of plasma ASN. In PD patients without point mutations in PRKN gene Parkin and ASN plasma levels increase until 2nd stage of disease in Hoehn and Yahr scale and during first 10 years of disease.

Analysis of the variations of PARK gene as well as plasma levels of ASN and Parkin may consist an additional diagnostic factor for PD.

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