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Iron Metabolism in Parkinson's Disease: DMT1 (-IRE) and TfR2 are regulated by microRNAs

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List of abbreviations

PD: Parkinson's Disease

ND: Neurodegeneration

SN: Substantia Nigra

TfR: Transferrin Receptor

DMT1: Divalent Metal Transporter 1

IRE: Iron Responsive Element

IRP: Iron Regulative Protein

miR, miRNA: MicroRNA

MPP^+: 1-methyl-4-phenylpyridine

WT: Wild Type

UTR : Untranslated Region

ISC: Iron-Sulfur Cluster

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Abstract

Parkinson's Disease is one of the most frequent human neurodegenerations. Motor symptoms of Parkinson's disease are the consequence of the destruction of nervous cells in the substantia nigra (SN). For many years it has been believed that the degeneration of nervous cells in SN in Parkinson's disease is related to an important increase in the concentration of iron. This excess of iron, which may initiate Fenton reaction, may be the cause of the oxidative stress leading to the death of nervous cells in PD. However, the mechanisms involved in iron accumulation remain unclear. Transferrin Receptor type 2 (TfR2) is a protein expressed on cell membrane end involved in the cellular iron uptake. It is not ubiquitously expressed like Transferrin Receptor type 1 but it is especially expressed in several tissues such as liver and recently, it has been discovered to be strongly involved in neuronal iron uptake in neurodegenerative disease. This suggests its potential involvement in the iron overload in the Substantia Nigra, a commonly observed phenomenon in Parkinson's Disease. The mRNA encoding for TfR2 does not contain the Iron Responsive Element (IRE) in its 3' UTR, so its regulation is surely non mediated by Iron Regulative Proteins (IRPs). Another gene/protein strongly involved in iron metabolism but not regulated by intracellular iron concentrations *via* IRPs is DMT1 -IRE. It is a divalent metal transporter so it could be

involved in PD iron excess. The aim of this thesis was to evaluate the expression of both the previously mentioned gene/proteins in a commonly used *in vitro* model of PD composed of SH-SY5Y treated with MPP^+ (1-methyl-4-phenylpyridinium), since they are not regulated by IRPs, and to answer the question: "Is supposable a regulation microRNA-mediated?".

The expression of DMT1 –IRE and TfR2 was evaluated at both mRNA and protein level in treated cells after 0, 24 and 48 hours from treatment with MPP^+. For both genes/proteins an increase in the expression was found in treated cells.

MicroRNA-221 was selected among two potential candidate microRNAs, restricted from a list of them resulted by bioinformatics prediction of microRNAs targeting the 3'UTR of TfR2. Using luciferase assay it was demonstrated the real ability of microRNA 221 in TfR2 3'UTR binding.

The function of microRNA-221 was verified in the chosen PD *in vitro* model. A similar experimental plan was used to verify the action of microRNA-Let-7d on the regulation of DMT1 –IRE isoform in the same PD cellular model.

In this thesis it is demonstrated that SH-SY5Y cells treated with MPP^+ show an increase in the expression of TfR2 and DMT1 -IRE respect to control cells (after 24 and 48 hours from treatment); at the same time, a significant reduction in the expression of microRNA-221 and of microRNA-Let-7d occurs. The over-expression of microRNA-221 in treated cells causes a decrease in the expression of TfR2, while when

cells are transfected with a vector containing microRNA-221 mutated in the binding site for the seed region, this microRNA loses the ability to bind the 3'UTR of TfR2 and its expression is completely restored. On the other hand, the over-expression of microRNA-Let-7d in treated cells causes a decrease in the expression of DMT1 -IRE, while cells transfected with a vector containing a mutated form of microRNA-Let-7d that loses the ability to bind the 3'UTR of TfR2, show a TfR2 expression restored and comparable with the expression in untreated cells.

These results suggest that microRNA-221 is involved in the iron metabolism fine regulation, acting specifically on TfR2 expression in the used Parkinson's Disease *in vitro* model and that microRNA-Let-7d is able to regulate the expression of DMT1 –IRE, a transporter that could be involved in the iron over-load in PD. Further studies could show the way forward to the therapeutic targeting of this pathway which could be directed to the down-regulation of the expression of both TfR2 and DMT1 -IRE in the dopaminergic neurons of the *Substantia Nigra* in PD. This findings could be important to obviate the need for the systemic ferrochelant therapy to which patients are often subjected as adjuvant treatment together with the specific therapy for Parkinson's Disease.

Introduction

1. Parkinson's disease

Parkinson's disease (also known as Parkinson's, Parkinson Disease or PD) is a degenerative disorder of the central nervous system (CNS) that often impairs the sufferer's motor skills, speech, and other functions (Jankovic J, 2008). Parkinson's disease belongs to a group of conditions called movement disorders. It is characterized by muscle rigidity, tremor, a slowing of physical movement (bradykinesia) and loss of a physical movement (akinesia) in extreme cases. The primary symptoms are the results of decreased stimulation of the motor cortex by the basal ganglia, normally caused by the insufficient formation and action of the dopamine, which is produced in the dopaminergic neurons of the brain (specifically the substantia nigra, SN). Secondary symptoms may include high level cognitive disfunction and subtle language problems. PD is both chronic and progressive.

PD is the most common cause of chronic progressive parkinsonism, a term which refers to the syndrome of tremor, rigidity, bradykinesia and postural instability. PD is also called "primary parkinsonism" or "idiopathic PD" (classically meaning having no known causes). While many forms of parkinsonism are idiophatic, "secondary" cases may result from toxicity most notably of drugs, head trauma, or other

medical disorders. The disease is named after English apothecary James Parkinson, who made a detailed description of the disease in his essay: "An Essay on the Shaking Palsy" (1987).

1.1 Classification

The term parkinsonism is used for a motor syndrome whose main symptoms are tremor at rest, stiffness, slowing of movement and postural instability. Parkinsonian syndromes can be divided into four subtypes according to their origin: primary or idiopathic, secondary or acquired, hereditary parkinsonism, and parkinson plus syndromes or multiple system degeneration (Jankovic J, 2008). Parkinson's disease is the most common form of parkinsonism and is usually defined as "primary" parkinsonism, meaning parkinsonism with no external identifiable cause (Samii A *et al*. 2004; Schrag A *et al*. 2007). In recent years several genes that are directly related to some cases of Parkinson's disease have been discovered. As much as this can go against the definition of Parkinson's disease as an idiopathic illness, genetic parkinsonism disorders with a similar clinical course to PD are generally included under the Parkinson's disease label. The terms "familial Parkinson's disease" and "sporadic Parkinson's disease" can be used to differentiate genetic from truly idiopathic forms of the disease (Davie CA, 2008).

PD is usually classified as a movement disorder, although it also gives rise to several non-motor types of symptoms such as sensory deficits

(Barnett-Cowan M, 2010), cognitive difficulties or sleep problems. Parkinson plus diseases are primary parkinsonisms which present additional features. They include multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration and dementia with Lewy bodies. In terms of pathophysiology, PD is considered a synucleinopathy due to an abnormal accumulation of alpha-synuclein protein in the brain in the form of Lewy bodies, as opposed to other diseases such as Alzheimer's disease where the brain accumulates tau protein in the form of neurofibrillary tangles (Galpern WR *et al*., 2006). Nevertheless, there is clinical and pathological overlap between tauopathies and synucleinopathies. The most typical symptom of Alzheimer's disease, dementia, occurs in advanced stages of PD, while it is common to find neurofibrillary tangles in brains affected by PD (Galpern WR *et al*., 2006). Dementia with Lewy bodies (DLB) is another synucleinopathy that has similarities with PD, and especially with the subset of PD cases with dementia. However the relationship between PD and DLB is complex and still has to be clarified (Aarsland D *et al*., 2009). They may represent parts of a *continuum* or they may be separate diseases (Aarsland D *et al*., 2009).

1.2 Signs and Symptoms

Motor

Four motor symptoms are considered cardinal in PD: tremor, rigidity, slowness of movement, and postural instability (Jankovic J, 2008).

- Tremor is the most apparent and well-known symptom. It is the most common; though around 30% of individuals with PD do not have tremor at disease onset, most develop it as the disease progresses. It is usually a rest tremor: maximal when the limb is at rest and disappearing with voluntary movement and sleep. It affects to a greater extent the most distal part of the limb and at onset typically appears in only a single arm or leg, becoming bilateral later (Jancovic J, 2008).
- Bradykinesia is another characteristic feature of PD, and is associated with difficulties along the whole course of the movement process, from planning to initiation and finally execution of a movement. Performance of sequential and simultaneous movement is hindered (Jancovic J, 2008). Bradykinesia is the most disabling symptom in the early stages of the disease (Samii A *et al*., 2004).
- Rigidity is stiffness and resistance to limb movement caused by increased muscle tone, an excessive and continuous contraction of muscles. In parkinsonism the rigidity can be uniform (lead-pipe rigidity) or ratchety (cogwheel rigidity) (Banich MT *et al*., 2011; Samii A *et al*., 2004; Jancovic J, 2008). The combination of tremor and increased tone is considered to be at the origin of cogwheel rigidity. Rigidity may be associated with joint pain; such pain being a frequent initial manifestation of the disease (Jancovic J, 2008). In early stages of Parkinson's disease, rigidity is often asymmetrical and it tends to affect the neck and shoulder muscles prior to the muscles of the face and

extremities. With the progression of the disease, rigidity typically affects the whole body and reduces the ability to move.

- Postural instability is typical in the late stages of the disease, leading to impaired balance and frequent falls, and secondarily to bone fractures. Instability is often absent in the initial stages, especially in younger people (Samii A *et al*., 2004; Jancovic J, 2008). Up to 40% of the patients may experience falls and around 10% may have falls weekly, with number of falls being related to the severity of PD.

Neuropsychiatric

Parkinson's disease can cause neuropsychiatric disturbances which can range from mild to severe. This includes disorders of speech, cognition, mood, behaviour, and thought. Cognitive disturbances can occur in the initial stages of the disease and sometimes prior to diagnosis, and increase in prevalence with duration of the disease (Jancovic J, 2008; Caballol N *et al*., 2007). The most common cognitive deficit in affected individuals is executive dysfunction, which can include problems with planning, cognitive flexibility, abstract thinking, rule acquisition, initiating appropriate actions and inhibiting inappropriate actions, and selecting relevant sensory information. Fluctuations in attention and slowed cognitive speed are among other cognitive difficulties. Memory is affected, specifically in recalling learned information. Nevertheless, improvement appears when recall is aided by cues. Visuospatial difficulties are also part of the disease (Caballol N *et al*., 2007).

A person with PD has two to six times the risk of suffering dementia compared to the general population. The prevalence of dementia increases with duration of the disease. Behavior and mood alterations are more common in PD without cognitive impairment than in the general population, and are usually present in PD with dementia. The most frequent mood difficulties are depression, apathy and anxiety. Psychotic symptoms, hallucinations or delusions occur in 4% of patients, and it is assumed that the main precipitant of psychotic phenomena in Parkinson's disease is dopaminergic excess secondary to treatment; it therefore becomes more common with increasing age and levodopa intake (Friedman JH, 2010).

Others

In addition to cognitive and motor symptoms, PD can impair other body functions. Sleep problems are a feature of the disease. Symptoms can manifest in daytime drowsiness, disturbances in REM sleep, or insomnia. Alterations in the autonomic nervous system can lead to orthostatic hypotension (low blood pressure upon standing), oily skin and excessive sweating, urinary incontinence and altered sexual function (Jancovic J, 2008). Constipation and gastric dysmotility can be severe enough to cause discomfort and even endanger health (Barichella M *et al*., 2009). PD is related to several eye and vision abnormalities (Armstrong RA, 2008). Changes in perception may include an impaired sense of smell, sensation of pain and paresthesia

(skin tingling and numbness). All of these symptoms can occur years before diagnosis of the disease (Jancovic J, 2008).

1.3 Causes

To date, despite decades of intensive study, the causes of PD remain unknown. Many evidences show that the disease is caused by a combination of genetic and environmental factors, which may vary from person to person.

In some people, genetic factors may play a role; in others, illness, an environmental toxin or other event may contribute to PD. Aging is surely an important risk factor. With a prevalence of approximately 1% at age 65, which rises to nearly 5% by age 85, PD is the second most common neurodegenerative disorder after Alzheimer's disease (de Lau LML *et al*., 2006; Van Den Eeden SK *et al*., 2003). The chemical or genetic trigger that starts the cell death process in dopamine neurons is the subject of intense scientific study. Generically we can divide Parkinson's causes in two different groups:

- **Genetic causes**

  PD traditionally has been considered a non-genetic disorder however at least between 5 and 10% of patients are now known to have monogenic forms of the disease (Lesage S *et al*., 2009). Other genes act as a risk factor for sporadic cases of the disease. To date, a number of specific genetic mutations causing PD have been discovered. Linkage studies and positional cloning strategies have identified mutations in a number

of genes that cause several monogenic autosomal-dominant or autosomal-recessive forms of the disorder. Mutations in at least five genes have been firmly linked to either dominant or recessive early-onset familial forms of PD. Additional loci segregating with inherited PD have been identified and the causative genes await identification. Known "PD genes" are α-synuclein, parkin, DJ-1, PTEN-induced kinase-1 (PINK1) and leucine-rich repeat kinase 2 (LRRK2)/Dardarin. Mutations in these genes result in cellular alterations that are similar to those seen in sporadic PD and may include impaired mitochondrial function and dynamics, increased oxidative stress (sensitivity) and abnormal protein aggregation (Figure 1).

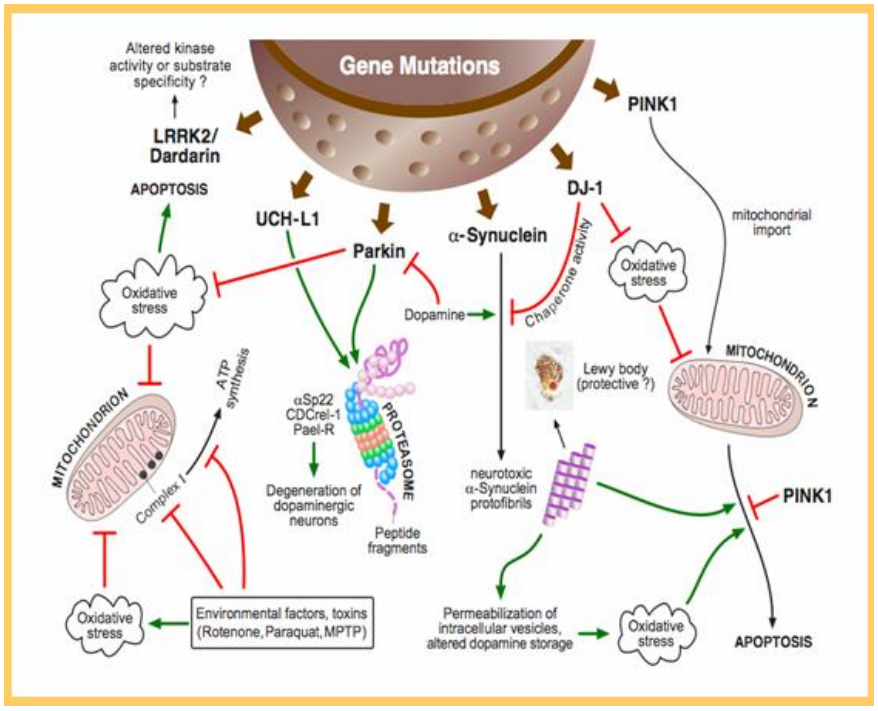

Figure 1. Genes involved in Parkinson's Disease.

Although most of these Mendelian forms of Parkinson's disease are rare, whole-genome association studies have more recently provided

convincing evidence that low-penetrance variants in at least some of these, but also in several other genes, play a direct role in the etiology of the common sporadic disease as well (Simon-Sanchez J *et al*., 2009). In Table 1 is reported a list of the most important genes involved in PD. The role of SNCA gene (encoding for alpha-synuclein) is central in the pathophysiology of PD, since alpha-synuclein protein is the main component of Lewy bodies, which are present in this and other diseases. Missence mutation of the gene and duplications or triplications of the locus containing it have been found in different groups of familial PD (Lasage S *et al*., 2009).

Locus	Position	Gene	Transmission
A			
PARK1	4q21-q23	SNCA (a-synuclein)	dominant
PARK8	12p11-q13	LRRK2	dominant
PARK2	6q25-q27	parkin	recessive
PARK6	1p36-p35	PINK1	recessive
PARK7	1p36	DJ-1	recessive
B			
PARK3	2p13	unknown	dominant
PARK9	1p36	unknown	recessive
PARK10	1p32	unknown	unknown
PARK11	2q36-q37	unknown	unknown
/	Xq	unknown	unknown
C			
PARK5	4p14	UCHL1	unknown
/	2q22-q23	NR4A2 (NURR1)	unknown
/	5q23	SNCAIP (synphilin-1)	unknown

Legend
A - Genes characterized by a conclusive involvement in PD, identified by "genome-wide" linkage studies
B - Other genes identified by a "genome-wide " approach, involved in PD
C - Genes probably associated to PD, identified by "candidate-gene" approach

Table 1. List of the most important genes involved in PD, chromosome position and tranmission.

- **Toxins**

Findings on environmental predisposing to the disease have remained in general incomnsistent. Most replicated relationship are in increased risk of PD in those living in rural environments and those expose to pesticides and a reduced risk in smokers (Davie CA, 2008). In a longitudinal investigation, individuals who were exposed to pesticides had a 70% higher incidence of PD than individuals who were not exposed (Ascherio A *et al*., 2006).

1.4 Hint of pathophysiology of Parkinson's Disease

Overview

The Basal Ganglia is an important group of structures in the Central Nervous System that function in the regulation and control of movement. These structures are nuclei that are interconnected with each other and with other cortical and subcortical structures. They use two separate pathways to send input received from the Sensorimotor Cortex through the various structures that make up the Basal Ganglia and back out to the Motor Cortex. They do this through the use of the neurotransmitters Glutamate, GABA, Enkephalin, Substance P, Acetylcholine, and Dopamine. The direct pathway results in a facilitation of a desired movement and the indirect pathway results in an inhibition of undesired movement. Dysfunction of these Basal Ganglia structures and pathways results in the movement disorder known as Parkinson's disease.

Functional Anatomical Review

The Basal Ganglia are a somatotopically organized grouping of nuclei located deep to the cortex that surround the Thalamus and are superior to the brainstem. They function in the processing of information in regards to the execution of movement. Its constituent parts are the Caudate, Putamen, Globus Pallidus, Subthalamic Nucleus (STN), and Substantia Nigra. The Caudate and Putamen make up the Striatum and are the main receptors for input to the Basal Ganglia from other areas. The Corpus Striatum is the same as the Striatum but it includes the Globus Pallidus, and the Lenticular Nucleus consists of the Putamen and Globus Pallidus. The Globus Pallidus has both an internal (GPi) and an external segment (GPe). The Substantia Nigra also has two parts -- the pars compacta (SNc) and pars reticulata (SNr). The GPe, STN, and SNc are involved in the internal processing within the Basal Ganglia pathways. The two main output nuclei are the SNr and GPi.

The Caudate is located most superior of the nuclei. It is also the largest forming the anterior portion of the Striatum and stretching back over and around the Lenticular Nucleus. Inferior and lateral to that is the Putamen, then the Globus Pallidus is internal to that. The Subthalamic Nucleus is located beneath the Thalamus as you probably inferred from its name. The Substantia Nigra is located in the Midbrain near the cerebral peduncles. There are two pathways within the Basal Ganglia: the direct pathway and the indirect pathway. The direct pathway facilitates intended movements and the indirect pathway inhibits

unintended movements. The direct pathway begins with the Striatum being excited by the Cortex. The Striatum then inhibits the internal segment of the Globus Pallidus and the Substantia Nigra pars reticulata using the neurotransmitters GABA or Substance P (Sub P). When these structures are inhibited they cannot inhibit the Thalamus rendering it free to fire and send excitatory input up to the Cortex, which facilitates movement. The indirect pathway begins with the Striatum being excited by the Cortex (just like the direct pathway). Then Striatal neurons send inhibitory input to the external segment of the Globus Pallidus using the neurotransmitters GABA or Enkephalin (enk). The Globus Pallidus external segment usually sends inhibitory input to the Subthalamic Nucleus using GABA, but if it is inhibited by the Striatum then it is unable to inhibit the Subthalamic nucleus leaving it free to fire. The Subthalamic Nucleus being uninhibited sends the only purely excitatory input within the Basal Ganglia pathways to the Globus Pallidus internal segment and the Substantia Nigra *pars reticulata*. These structures then inhibit the Ventral lateral nucleus (VL) and Ventral anterior nucleus (VA) of the Thalamus making it unable to send excitatory input to the Cortex and thus indirectly inhibiting the Motor Cortices, which inhibits movement. The Substantia Nigra pars compact plays a key role in the balance of direct versus indirect pathway. If functioning correctly the Substantia Nigra *pars compacta* has the overall effect of facilitating intended movement and inhibiting unintended movement. It does this by exciting the direct pathway and inhibiting the indirect pathway

through release of the neurotransmitter Dopamine. Striatal neurons of the direct pathway have D1 receptors which are excited by Dopamine, whereas Striatal neurons of the indirect pathway have D2 receptors which are inhibited by Dopamine. The Substantia Nigra *pars compacta* also sends excitatory input directly to the Cortex again using Dopamine. Parkinson's is a result of a disruption between the balance of the direct versus the indirect pathway. This balance is tipped more towards the indirect pathway resulting in less facilitation and more inhibition of movement. This disruption is caused by lesions of the Substantia Nigra *pars compacta*. This death of neurons in the pars compacta means that they can no longer release dopamine and excite the Striatal neurons in the direct pathway and inhibit the Striatal neurons in the indirect pathway. The *pars compacta* also can no longer excite the Cortex directly.

Now due to the over-activity of the indirect pathway we see the signs of Parkinson's disease being exhibited. We understand why these are the symptoms because we know how the pathways within the Basal Ganglia function. All of the symptoms reflect the increase in inhibition of movement and the decrease in facilitation.

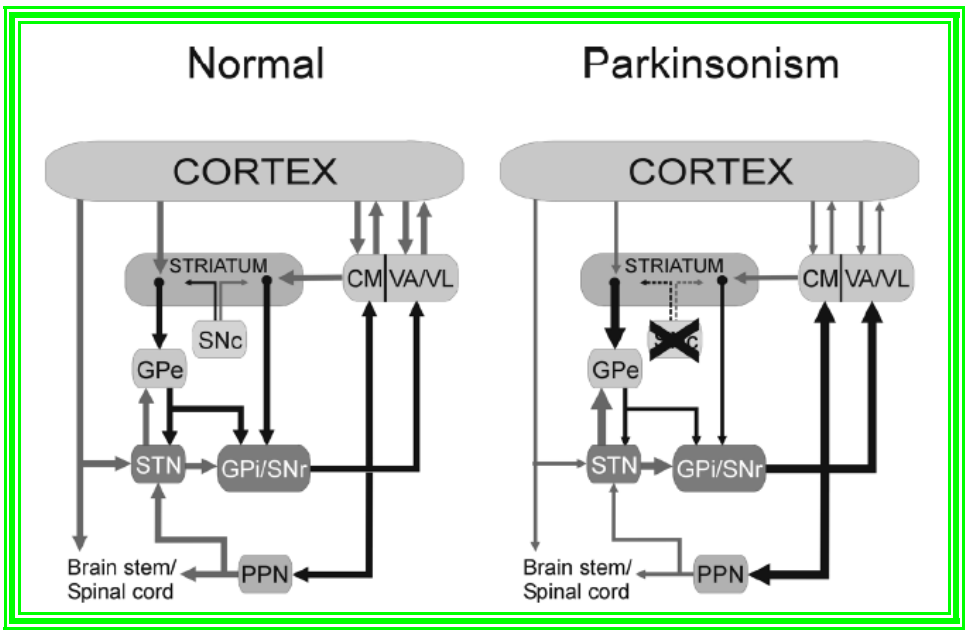

Figure 2. : Pathophysiology of Parkinson's Disease. Simplified schematic diagram of the basal ganglia-thalamocortical circuitry under normal conditions (left) and rate changes in parkinsonism (right). Inhibited connections are shown as *filled arrowds*, excitatory connections as *open arrows*. The principal imput nuclei of the basal ganglia, the striatum and the STN (subthalamic nucleus), are connected to the output nuclei, Gpi and SNr. Basal ganglia output is directed at several thalamic nuclei (VA/VL and centromedian nucleus of thalamus shown as CM) and at brain stem nuclei (pedunculopontine nucleus shown as PPN and others). In parkinsonism, dopaminergic neurons in the SNc degenerate, which results via a cascade of change in the other basal ganglia nuclei, in increased basal ganglia output from Gpi and SNr. This, in turn, is thought to lead to inhibition of related thalamic and cortical neurons.

2. Oxidative stress in Parkinson's Disease

Nigral dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine gives rise to various molecules that can act as endogenous toxins if not handled properly (Figure 3). Dopamine can auto-oxidize at normal pH into toxic Dopamine-quinone species, superoxide radicals and hydrogen peroxide. Alternatively, it can be enzymatically deaminated by monoamine oxidase (MAO) into the non-toxic metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and

hydrogen peroxide (Maker HS *et al*., 1981). Superoxide is not a highly reactive molecule, but it can be converted to hydrogen peroxide by superoxide dismutase, or into labile peroxinitrite radicals in the presence of nitric oxide. Hydrogen peroxide is also innocuous, but can be broken down into cytotoxic hydroxyl radicals in a reaction that is catalysed by iron, the levels of which are higher in the substantia nigra than in other brain regions. Reactive oxygen species lead to functional alterations in proteins, DNA and lipids. Lipid damage, in turn, leads to loss of membrane integrity and increased permeability to ions such as calcium, which can promote excitotoxicity (Halliwell, B *et al*., 1992) (Figure 3). As cytoplasmic dopamine can quickly form reactive oxygen species, dopamine that has been synthesized or transported into the cell from the extracellular space has to be rendered harmless by rapid storage into small synaptic vesicles. By virtue of their low pH and the absence of MAO, these structures provide a stable environment for dopamine. Under normal conditions, efficient dopamine sequestration is probably the main pathway by which nigral cells protect themselves from the deleterious effects of dopamine oxidation. In Parkinson's disease, however, nigral cells seem to be under a heightened state of oxidative stress, as indicated by elevations in by-products of lipid, protein and DNA oxidation, and by compensatory increases in antioxidant systems (Mecocci P *et al*., 1993; Martilla RJ *et al*., 1988; Ben-Shachar D *et al*., 1991; Riederer *et al*.,1989; Jelliger K *et al*., 1992; Dexter DT *et al*., 1989).

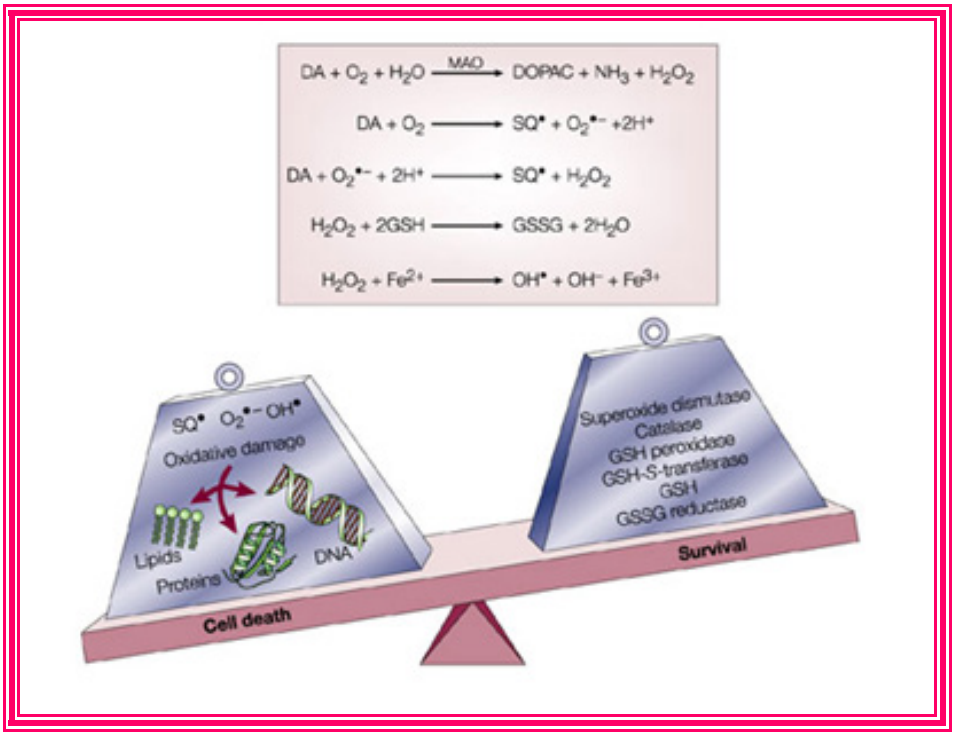

Figure 3. Cytoplasmic dopamine can form reactive oxygen species. An imbalance between the production and elimination of reactive oxygen species could contribute to the pathogenesis of Parkinson's disease and other neurodegenerative disorders. Metabolism of dopamine (DA) leads to the formation of several cytotoxic molecules, including superoxide anions (O_2^-), dopamine–quinone species (SQ) and hydroxyl radicals (OH). Dopamine breakdown can occur spontaneously in the presence of iron, or can be catalysed by monoamine oxidase (MAO) in a reaction that generates hydrogen peroxide (H_2O_2). Even though hydrogen peroxide is not damaging to cells, the formation of hydroxyl radicals by the Fenton reaction can lead to cytotoxicity. Normally, cells scavenge these deleterious molecules using several antioxidant systems. For instance, glutathione (GSH) peroxidase detoxifies hydrogen peroxide using reduced glutathione. Oxidized glutathione (GSSG) can be reduced by GSSG reductase and reused. GSH-S-transferase converts electrophilic centres of various potentially toxic compounds to thioether bonds. Superoxide dismutase converts superoxide to hydrogen peroxide. Catalase, in turn, converts hydrogen peroxide to molecular oxygen and water. In Parkinson's disease, however, an abnormal increase in the production of reactive oxygen species might tilt the balance between production and elimination, leading to enhanced oxidative stress. DOPAC, 3,4-dihydroxyphenylacetic acid.

2.1 Role of Iron in Parkinson's disease

Iron may play a deleterious role in living cells by triggering of the oxidative stress via Fenton reaction (Halliwell B, 1989). Under physiological conditions this reaction takes place in cells without causing any damage because the amounts of the produced free radicals are not sufficient to start the stress. However an excess of divalent iron or a defect in annihilation of the radicals by scavengers may start the cascade of noxious events. The concentration of the total iron in parkinsonian and control substantia nigra was assessed by many authors with the use of various procedures (Gałazka-Friedman J *et al*., 1997; Friedman A *et al*., 2007). Although according to many studies, most of iron is bound to ferritin, a controversy exists in the literature how much iron could be bound to neuromelanin. Some authors found iron bound to neuromelanin (Jellinger K *et al*., 1992), others did not (Hirsch EC *et al*., 1991). Another question is related to the placement of iron in neurons or in glia. In an interesting experiment using X-ray microanalysis an increase of iron concentration in individual neurons of SN was detected (Oakley AE *et al*. 2007). It may be concluded that the distribution of iron in SN is not homogenous. This also may affect the results obtained by various laboratories. The damage caused by iron is related to the oxidative stress triggered by Fenton reaction:

$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$

Iron is a strong prooxidant that catalyzes the production of hydroxyl radicals (Figure 4) leading cells to the death because of the severe

alterations of DNA, proteins, lipids and mithocondria. Although literature shows several contradiction regarding iron presence in the substantia nigra, the commonly reported increase of iron content in it suggests either an increase in iron penetration in the affected brain regions in patients with PD or a poor elimination of iron.

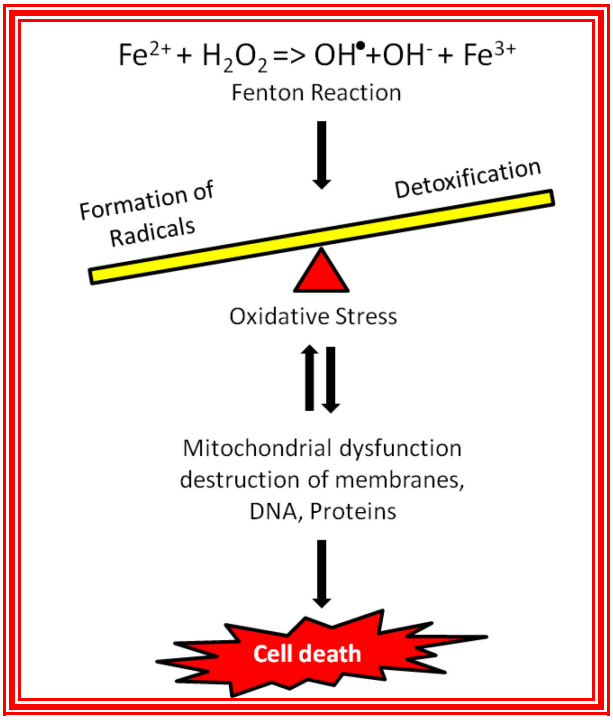

Figure 4. Simplified scheme of iron-induced pathological cascade involved in neurodegeneration with brain iron accumulation associated with parkinsonian syndromes. Up to now, the hypothesis is that both, a genetic predisposition and endo- and/or exotoxins, may lead to enhanced iron levels that promote, primarily by means of the Fenton reaction, the formation of radicals that overwhelm the detoxification capacity of the cell, resulting in destruction of organelles and ultimately cell death.

When the amount of iron exceeds the detoxification systems of the cell, increased levels of iron, especially Fe^{2+} enhance the Fenton reaction resulting in an amplification of oxidative stress (Riederer P *et al*., 1993).

This may induce a vicious circle as oxidative stress may increase the levels of free iron and so on.

Oxidative stress is a common feature in many neurodegenerative disorders. The origin of the general oxidative stress in neurodegeneration is not fully understood and it is still unclear if the rise in iron concentration in the brain of patients with PD coud be a cause or an effect of PD.

3. General Iron Metabolism

Iron is an essential metal for the organism because of its unparalleled versatility as a biological catalyst. Consequently, iron is a crucial element required for growth. However, the very chemical properties of iron that allow this versatility also create a paradoxical situation, making acquisition by the organism very difficult. Indeed, at pH 7.4 and physiological oxygen tension, the relatively soluble iron (II) (Fe^{2+}) is readily oxidized to iron (III) (Fe^{3+}), which upon hydrolysis forms insoluble ferric hydroxides. As a result of this virtual insolubility and potential toxicity because of redox activity, iron must be constantly chaperoned. In fact, specialized molecules for the acquisition, transport, and storage of iron in a soluble, nontoxic form have evolved to meet the organism's iron requirements. Because of its redox properties, iron can catalyze the production of reactive oxygen species (ROS) that can be highly toxic (Eaton JW *et al*., 2002). Therefore, under normal physiological conditions, iron is specifically transported in the blood by

diferric transferrin (Tf) (Klausner RD *et al*., 1983; Iacopetta BJ *et al*., 1983). All tissues acquire iron by the binding of Tf to the transferrin receptor 1 (TfR1), with this complex then being internalized by receptor-mediated endocytosis (Klausner RD *et al*., 1983; Iacopetta BJ *et al*., 1983) (Figure 5A).

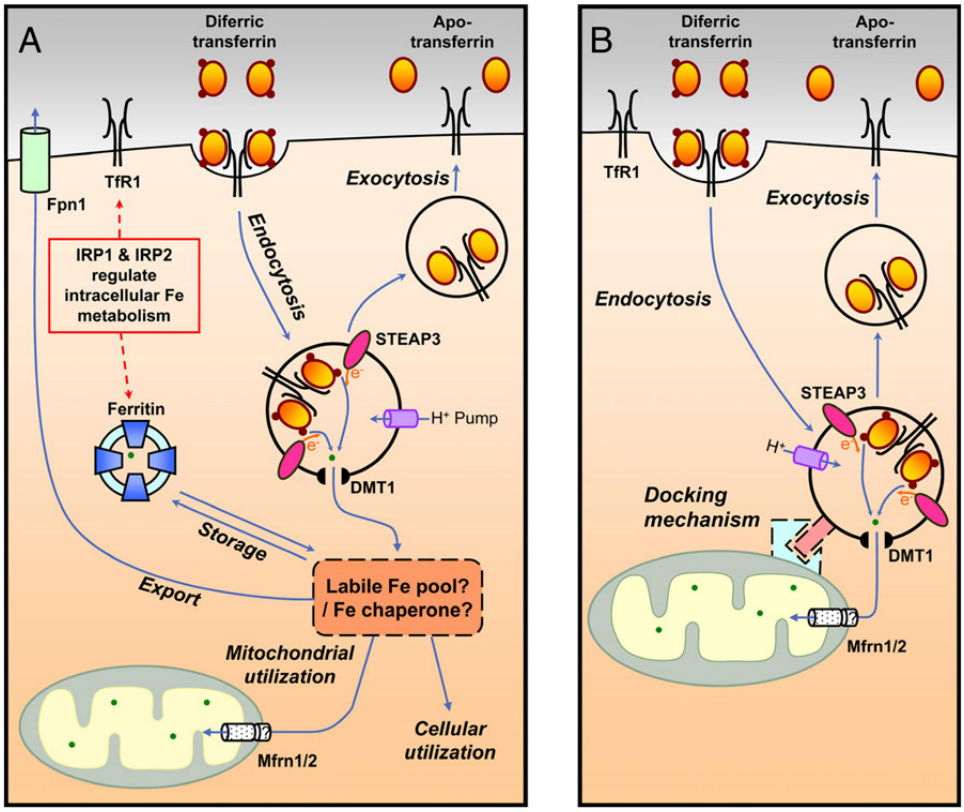

Figure 5. Schematics of cellular iron uptake. (A) The process or iron uptake and utilization. (B) The "kiss and run" hypothesis.

Within the endosome, the affinity of Tf for iron is decreased by the low pH generated through the activity of a proton pump (Ponka P,1997; Dunn LL *et al*., 2007). Importantly, the TfR1 facilitates liberation of iron from Tf in the pH range attained by the endosome (pH 5–5.5) (Bali PK *et al*., 1991). In vitro, iron release from Tf requires a "trap", such as

pyrophosphate (Bali PK *et al*., 1991), but a physiological chelator serving this role has not been identified. In erythroid cells, once iron(III) (Fe^{3+}) is released from Tf in the endosome, it is thought to be reduced to iron(II) (Fe^{2+}) by a ferrireductase in the endosomal membrane known as the six-transmembrane epithelial antigen of the prostate 3 (STEAP3) (Ohgami RS *et al*., 2005; Sendamarai AK *et al*., 2008) (Figure 5A). After this step, Fe^{2+} is then transported across the endosomal membrane by the divalent metal transporter-1 (DMT1) (Fleming MD *et al*., 1998) and forms, as generally believed, the cytosolic low Mr (Relative Molecular Mass) labile or chelatable iron pool (Jacobs A, 1977). This pool of iron is thought to supply the metal for storage in the cytosolic protein ferritin and for metabolic needs, including iron uptake by the mitochondrion for heme and Iron-Sulfur clusters (ISCs) synthesis. Iron can also be released from the cell by the transporter ferroportin1 (Ganz T, 2007) (Figure 5A).

Ferroportin1 expression can be regulated by the hormone of iron metabolism, hepcidin. Hepcidin is a key regulator of systemic iron metabolism (Ganz T, 2007) and is transported in the blood bound to α2–macroglobulin (alpha 2-macroglubulin) (Ganz T, 2007). Hepcidin secretion by the liver is stimulated by high iron levels and also inflammatory cytokines, such as interleukin-6 (Ganz T *et al*., 2009). The chelatable iron pool is thought to control the activity of IRPs-1 and -2 (Figure 6). The IRPs are RNA-binding proteins that bind to IREs in the 3’- and 5’-untranslated regions in mRNAs of molecules playing

crucial roles in the uptake, utilization, export, and storage of iron (e.g., TfR1, ferritin, etc.) (Hentze MW *et al*., 2004). IRP-1 performs two functions: (1) regulating iron homeostasis via binding IREs and (2) having cytosolic aconitase activity when containing an [4Fe-4S] cluster (Hentze MW *et al*., 1996). IRP-2 is thought to be the principal RNA-binding protein in vivo and is regulated by iron-dependent proteasomal degradation (Cairo G *et al*., 2007; Meyron-Holtz EG *et al*., 2004; Meyron-Holtz EG *et al*., 2004). Once iron is transported out of the endosome via DMT1, it enters the chelatable (and stored in ferritins) or labile iron pool (Figure 5A) or, alternatively it is deliverd to mithocondria in a still unclear way. A direct transfer of iron from Tf to the mitochondrion was proposed to occur (Isobe K *et al*., 1981; Ponka P *et al*., 1976). This idea has developed in more recent years and has led to the "kiss and run" hypothesis (Ponka P, 1997) (Figure 5B). This model suggests that a direct transfer of iron from the Tf-containing endosome to the mitochondrion occurs, by-passing the cytosol (Ponka P, 1997; Zhang AS *et al*., 2005).

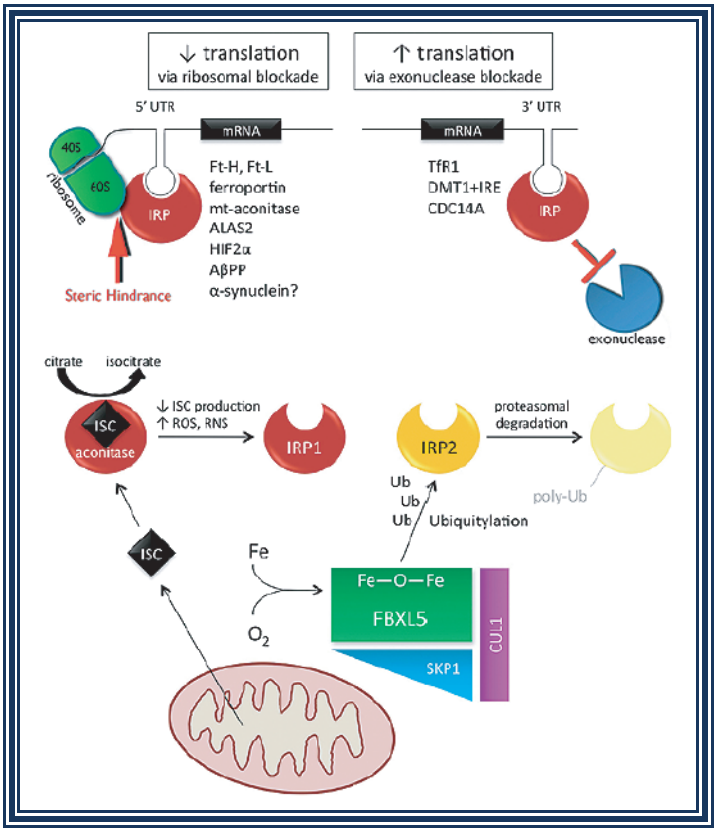

Figure 6. Iron metabolism regulation by IRPs. Iron regulatory proteins control cellular iron homeostasis. A) Transcripts containing iron-regulatory elements (IREs) in either their 5′ or 3′ Untranslated Regions (UTR) are bound by iron regulatory proteins (IRPs) when iron levels are perceived to be low. IRP binding to 5′ IREs provides steric hindrance to ribosomal machinery, leading to decreased translation of the transcript. IRP binding to 3′ IREs blocks the action of exonucleases, thereby protecting the transcripts from degradation and increasing translation. Transcripts that contain IREs in either their 5′ or 3′ UTRs are listed. Note: There are four isoforms of DMT1 mRNA, only some of which contain 3′ UTR IREs. Abbreviations: ferritin-H/-L, Ft-H/-L; mitochondrial aconitase, mt-aconitase; aminolevulinic acid synthase 2, ALAS2; hypoxia inducible factor 2*α*, HIF2 *α*; amyloid *β* precursor protein, A*β*PP; transferrin receptor 1, TfR1; divalent metal transporter with IRE, DMT1+IRE; cell division cycle 14 homolog A, CDC14A. B) When it contains a functional ISC, IRP1 (red) functions as a cytosolic aconitase (converting citrate to isocitrate); in the absence of a functional ISC it is converted to an IRE-binding protein. The status of the ISC can change in response to decreased ISC production or increased ISC disruption by reactive oxygen species (ROS). IRP2 (yellow) is a constitutive IRE-binding protein that is regulated by proteasomal degradation. When cellular iron and oxygen levels are adequate, FBXL5 (green) can complex with SKP1 (blue) and CUL1 (purple) to form a functional E3 ubiquitin ligase that tags IRP2 for degradation (light yellow).

3.1 Iron Metabolism and its "essential proteins"

Although it is strongly incorrect use the term "essencial" referred to only two proteins involed in iron metabolism, in this context it is necessary spent few words about the previously described Divalent Metal Iron Transporter DMT1 and the Transferrin Receptors:

- ***DMT1***

The divalent metal transporter 1 (DMT1), also known as natural resistance-associated macrophage protein 2 (NRAMP 2), and divalent cation transporter 1 (DCT1), is a protein that in humans is encoded by the *SLC11A2* (solute carrier family 11, member 2) gene (Vidal S *et al*., 1995). DMT1 represents a large family of orthologous metal ion transporter proteins that are highly conserved from bacteria to humans (Au C *et al*.,). As it's name suggests, DMT1 binds a variety of divalent metals including cadmium (Cd^{2+}) and copper (Cu^{2+}), however it is best known for it's role in transporting ferrous iron (Fe^{2+}); DMT1 expression is regulated by body iron stores to maintain iron homeostasis. DMT1 is also primary in the absorption and transport of manganese (Mn^{2+}). DMT1 expression in the brain may increase with age (Ke Y *et al*., 2005), increasing susceptibility to metal induced pathologies. DMT1 expression is found to be increased in the substantia nigra of Parkinson's patients and in the ventral mesencephalon of animal models intoxicated with 1-

methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) - a neurotoxin widely used experimentally to produce Parkinsonian symptoms.

Two mRNA isoforms differ in the 3' UTR: DMT1 +IRE has an IRE (Iron Responsive Element) but DMT1-IRE lacks this feature. The +/-IRE proteins differ in the distal 18 or 25 amino acid residues after shared identity for the proximal 543 residues. A major function is serving as the apical iron transporter in the lumen of the gut. The +IRE isoform appears to have that role. Another role is endosomal exit of iron. Some evidence indicts the -IRE isoform for this function. As a consequence, while the +IRE isoform can be regulated in its expression by the IRPs proteins, this can not happens for the –IRE isoform.

- ***TfRs***

Transferrin receptor (TfR) is a carrier protein for transferrin. It is needed for the import of iron into the cell. It imports iron by internalizing the transferrin-iron complex through receptor-mediated endocytosis. There are 2 different genes encoding for two different forms of Transferrin Receptors: (1) TfR1 (also called TfRC) is expressed quite ubiquitously and contains the IRE sequence in the 3'UTR of the mRNA encoding for it, (2) TfR2 is expressed less ubiquitously respect to TfR1. It is the principal Transferrin Receptor expressed, for exemple in the liver but a recent study (Mastroberardino PG *et al*., 2009) shows its strong expression in dopaminergic neurons in Parkinson's disease. Even

in this case, while TfR1 contains an IRE sequence in the 3'UTR of the mRNA encoding for it, TfR2 can not be regulated by IRPs.

The previously described observations arise an important question: **is it supposable that proteins such as DMT1 –IRE and TfR2, so strongly involved in iron uptaking and homeostasis are regulated by a mechanism microRNA-mediated?**

Few years ago, our group demonstrated that that in erythroid cells, DMT1-IRE expression is under the regulation of miR-Let-7d. DMT1-IRE and miR-Let-7d are inversely correlated with CD34(+) cells, K562 and HEL cells during erythroid differentiation. Moreover, overexpression of miR-Let-7d decreases the expression of DMT1-IRE at the mRNA and protein levels in K562 and HEL cells. MiR-Let-7d impairs erythroid differentiation of K562 cells by accumulation of iron in the endosomes (Andolfo I *et al*., 2010).

To date, no information regarding a putative microRNA-mediated mechanism of regulation of TfR2 is available in literature.

4. MicroRNAs (miRNAs) in brief

In recent years, our understanding of miRNAs has grown from the discovery of a single genetic oddity in worms to the recognition of an entirely new class of regulatory molecule with thousands of members (Griffiths-Jones S *et al*.,2008). The significance of miRNAs in normal development and cellular function is underscored by mounting evidence that misregulation of specific miRNA pathways is associated with complicated health afflictions, including cancer, heart disease and neurological disorders (Hebert SS *et al*., 2009; Latronico MV *et al*., 2009; Negrini M *et al*., 2009). miRNAs are intertwined in complex regulatory pathways in plants as well5 and represent one of the most plentiful classes of gene regulators in multicellular organisms. Production of the functional, ~22-nt mature miRNA involves multiple processing steps (Chen X, 2009; Davis BN *et al*., 2009; Winter J *et al*., 2009) (Figure 7). The general miRNA biogenesis pathway begins with synthesis of a primary transcript by RNA polymerase II (Pol II). Housed within the primary transcript is the hairpin precursor, which contains the sequence destined to be the mature miRNA in one arm of the stem. In animals, the Microprocessor complex, minimally composed of the Drosha RNase (RNase) and its RNA binding partner Pasha (also called DGCR8), releases the miRNA precursor from the primary transcript. Exportin-5 delivers the precursor to the cytoplasm for final processing by the Dicer RNase and its double-stranded RNA binding cofactor TRBP (also called loquacious, Loqs). After loading onto Argonaute, one strand

of the resulting partial duplex, designated the guide, is preferentially retained.

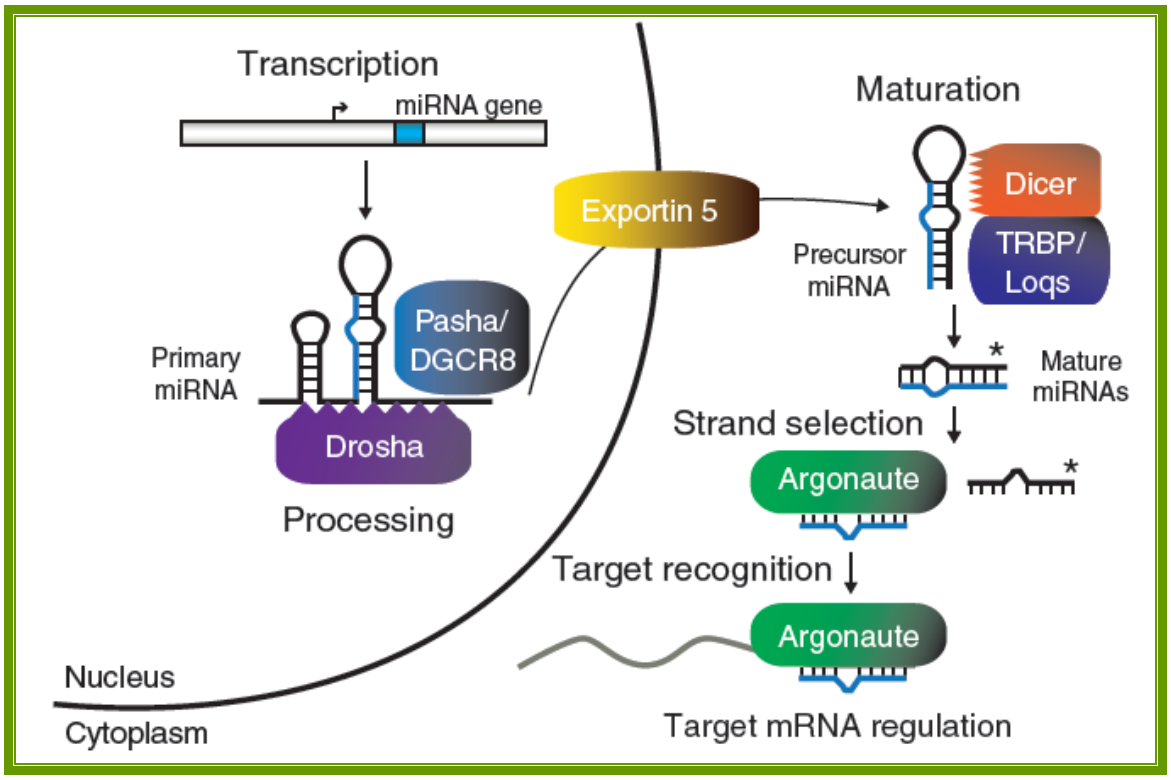

Figure 7. A general model of miRNA biogenesis and function. After synthesis by RNA polymerase II, miRNA primary transcripts are recognized by Pasha/DGCR8 and Drosha, which excises the hairpin precursor. Exportin 5 delivers the miRNA precursor to Dicer and its RNA binding partner, TRBP/Loqs, for final processing to the mature 22-nt miRNAs. One strand is selected for stable association with Argonaute, where it serves as a guide to target and regulate specific mRNAs.

This multistep pathway is shared in plants with a few exceptions: the Dicer-like (DCL) proteins catalyze both the primary and precursor processing steps in the nucleus, where the mature miRNA forms a complex with Argonaute and is transported to the cytoplasm. miRNAs

serve as guides to direct the Argonaute complex to target mRNAs through complementary base-pairing (Chen X, 2009; Chekulaeva M *et al*., 2009). Typically, target recognition results in destabilization or translational repression, either of which ultimately silences gene expression. Accumulation of a specific miRNA is dependent on the rates of transcription, processing and decay. Similar to the expression of many protein-coding genes, expression of miRNA primary transcripts is subject to regulation by specific transcription factors and chromatin marks (Chen X, 2009; Davis BN *et al*., 2009; Winter J *et al*., 2009). Control of each processing step has also emerged as a key determinant of functional miRNA expression. The first global analysis of primary and mature miRNA levels revealed that extensive post-transcriptional regulation is involved in cellular miRNA homeostasis (Thomson JM *et al.,* 2006). Several examples of proteins and mechanisms that govern processing of specific miRNAs are detailed in recent reviews (Davis BN *et al*., 2009; Winter J *et al*., 2009).

4.1. MicroRNAs and Neurodegenerative Diseases

"Neurodegeneration" is a term that has been used to refer to differing topics. Abundant nerve cell death occurs in the course of normal brain development, and many pediatric neurological diseases are characterized by pathological degeneration of neurons and/or muscles. Phenomena involving nerve cell death also occur in animal models and cells in culture. In the course of NDs, neurons lose their connections and die prematurely. There are six general ideas that are relevant at least circumstantially to how microRNA (miRNA) biochemistry may interact with the pathogenesis of Neurodegenerative Diseases (NDs):

1) Most NDs are not inherited in patterns that reflect simple Mendelian genetics. Most prevalent subtypes of NDs, such asAD and PD, are inherited in a manner termed "sporadic" (as opposed to "familial"), influenced by alleles with limited genetic penetrance, or are caused by genetic and/or environmental influences as yet uncharacterized. These facts may shift the focus of ND genetic studies away from "traditional" genes which constitute only ~1%–2% of human DNA, and towards the other ~50% of transcribed DNA about which we are mostly ignorant.

2) Brains of human ND patients show evidence of chronic stress. ND brains show evidence of considerable cellular stress. Although this may be expected in disease processes that last for long periods and involve abundant cell death, this response may prove contributory

because the stress response itself could play a role in the "cascade" effect (see below) that result ultimately in neurological deficits.

3) NDs may be exacerbated in the clinicobiological sense by the aberrant stimuli of developmental pathways. Some biochemical pathways that are up-regulated during normal brain development, but down-regulated in normal adulthood, are then aberrantly up-regulated again in the course of NDs. These include pathways involved in cell–cell signaling, cell division, neuroplasticity and apoptosis (Arendt T, 2005; Bothwell M *et al*., 2000; Nguyen MD *et al*., 2002). In the case of the microtubule-associated protein tau (MAPT), there is an mRNA splicing variant that is more highly represented both in early development and in some neurodegenerative diseases (Kar A *et al*., 2005; Nelson PT *et al*., 2007). Furthermore, the developmentally up-regulated phosphorylation of MAPT is also up-regulated in AD neurofibrillary pathology (Goedert M, 2004; Stoothoff WH *et al*., 2005). These are only a few examples of how the up-regulation of developmental pathways in the adult milieu may contribute to the pathological progression of some NDs.

4) RNA is pathologically altered in NDs RNA biochemists are well aware that RNA is labile in even tightly controlled circumstances. In the course of some NDs, brain RNAs becomes pathologically altered (Markesbery WR *et al*., 2007; Nelson PT *et al*., 2007; Smith MA *et al*., 2000). These changes include aberrant RNA oxidation, RNA

degradation, altered RNA splicing and ribosomal changes which cause mRNA translational frame-shifting abnormalities.

5) Human NDs are chronic conditions that last for decades rather than just for several years. Recent studies have established the surprising chronicity of the processes underlying NDs. UsingAD as an example, the time from patients' clinical diagnosis to death is typically ~8 years (Williams MM *et al*., 2006). However, the underlying pathological processes of AD occur over many decades. Persons at risk for developingAD in their seventh or eighth decade already show brain metabolic abnormalities by PET scan even in their third decade of life (103 Reiman EM *et al*., 2004; Scarmeas N *et al*., 2005). These are important data because they show that ND pathology involves a longevolving shift in cellular equilibrium, which could be "tipped" even by subtle genetic and/or environmental influences.

6) Pathogenetic mechanisms of ND involve multiple distinct Steps. Most NDs are probably not just a "one-hit" phenomenon, but rather they probably involve a sequential progression of pathological processes that result collectively in neuronal death and/or compromised connectivity.

These six features of NDs dovetail on some of the known characteristics of miRNA biochemistry in the brain. As described below, miRNAs derive from "non-traditional genes"; are related to pathways of cellular stress and neurodevelopment; may be altered by the changes seen in ND

brains such as oxidation; and may ultimately contribute to step(s) that culminate in chronic brain diseases.

One unexpected characteristic of some human miRNAs is their very high RNA copy number per cell. Particular miRNAs can be represented by 1000 – 30000 copies per cell or more (Allawi HT *et al*., 2004; Lim LP *et al*., 2003). By comparison, the large majority of mRNAs typically number less than 100 copies per cell (Carter MG *et al*., 2005; Sheng HZ *et al*., 1994). If miRNAs participate in gene expression regulation and are represented in high numbers per cell, how extensive is the cellular impact of miRNAs? Numerous studies have now established that miRNAs can have a profound cellular impact by dint of sheer biochemical promiscuity: each miRNA can regulate hundreds, thousands or more mRNA targets. This was first indicated by predictive , which showed that evolutionarily conserved "miRNA recognition elements" are present in the 3' untranslated region (3'UTR) of very many mRNAs per miRNA (Bentwich I, 2005; John B *et al*., 2004; Kiriakidou M *et al*., 2004; Lewis BP *et al*., 2003; Lewis BP *et al*., 2005; Maziere P *et al*., 2007). Hence, these small non-coding RNAs, which are present in very high gene copy numbers and which work through a powerful translational regulation paradigm, can modify an exceptionally large number of genes.

4.2. MicroRNAs in cellular stress

Cellular stress is a conspicuous feature of ND brains. A detailed catalog of the many roles now thought to be played by miRNAs in stress is beyond the scope of this review. However, miRNAs' expressions have been shown to change dramatically in response to cold and heat stress, hypoxia, oxidative stressors, arsenic, irradiation, nutrient deprivation and other stresses (Ambros V, 2003; Chiou TJ, 2007; Dresios J *et al*., 2005; Ishii H *et al*., 2006; Lukiw WJ *et al*., 2007; Marsit CJ *et al*., 2006; Rocha S, 2007; van Rooij E *et al*., 2006; Sunkar R *et al*., 2007). Typically, in stress conditions, a subset of miRNAs are increased, and a subset are decreased. The subset of miRNAs that are altered in one stress condition and in one cell type are not necessarily related to the miRNAs which change expression upon a different stressful stimulus. The biochemical effects of miRNAs during cellular stress can change dramatically. For example, stress may alter a miRNA : mRNA interaction to produce translational up-regulation of target mRNAs (Bhattacharyya SN *et al*., 2006; Huang J *et al*., 2007), which is the opposite of "canonical" miRNA activity. This has prompted speculation that miRNAs may constitute a "safeguard against turmoil", by altering globally the parameters of translational machinery in the cells during stress (Leung AK *et al*.,2007). **In summary, during any conditions that are attended by cellular stress, such as NDs, it can be expected that miRNA expression patterns will change, which would be expected in turn to alter the translation of a large number of**

different cellular mRNAs. Most of the details of how miRNAs participate in neurodegeneration have yet to be worked out. Hence, at this point, one may consider that the current research corpus is mostly a platform for future gains. Figure 8 shows some of the possibilities related to the interactions between miRNAs and NDs.

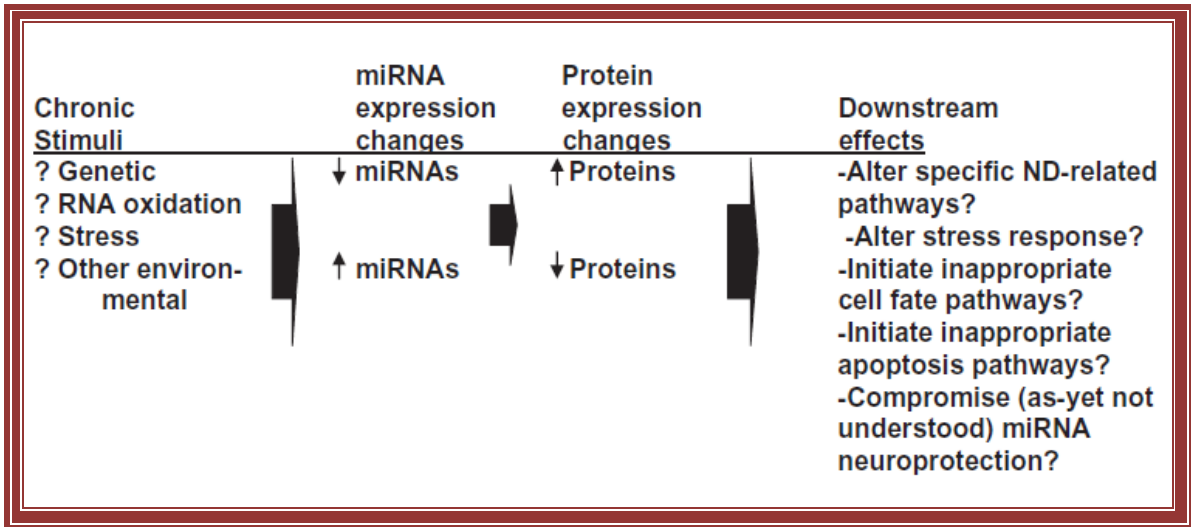

Figure 8. A schematic diagram about some roles miRNAs may play in NDs. The brains of persons at risk for NDs are subjected to chronic stimuli that can perturb specific and/or global miRNA expression. Either the aberrant stimulation or inhibition of miRNA expression may be associated with pathways involved in NDs or neuroprotection.

5. Aim of the study

In human brain the gratest amount of iron is localized in basal ganglia (Riederer *et al.*,1989), moreover, in old age a strong increase of its concentration is found in both humans and animal models (Zecca *et al.*, 2001; Hahn *et al.*,2009). The main feature of many neurodegenerative diseases is the difficoulty in the regulation and keeping of iron homeostasis. In Parkinson's Disease high concentration of iron have been observed in substantia nigra, a sub-region inside basal ganlia, subject to dopaminergic neurodegeneration which during the outcome of the disease is associated with motor disfunction. This increase can lead to unbalance and consequently iron overload determining the inability of neurons and other brain cells inside basal ganglia to try to avoid this toxic overload. Iron role in the phathogenesis of PD has been widely studied in the last two decades. Many scientific evidences confirm that among the causes iron can interact and interfere with normal dopaminergic functions or other features associated to PD (Pezzella *et al*., *1997*; Hashimoto *et al*., 1999). Alterations of one of the many components involved in the delicate balance between iron cellular transport and iron absorption and storage can cause a loss of its homeostasis and contribute to dopaminergic neurodegeneration. On these basis, great part of scientific literature questioned on which could be the mechanism that regulates genes involved in iron metabolism dysregulation. As explained before, TfR2 is mainly expressed in dopaminergic neurons of substantia nigra in

PD (Mastroberardino *et al*., 2009), moreover its post-transcriptional expression is not regulated by IRPs as it does not contain an IRE at the 3'UTR of the mRNA encoding for it. Being TfR2 a receptor expressed on cell membrane, involved in iron uptake, and being Parkinson's Disease characterized by iron overload, this study is oriented to investigate about the possible regulation of TfR2 expression by microRNAs. This hypothesis is supported by the evidence that other genes involved in iron metabolism are actually regulated by microRNAs. In fact, from a previously published study, developed in our laboratory, it has been demonstrated that Let-7d microRNA is able to regulate the expression of DMT1 –IRE transporter during erythroid cell differentiation (Andolfo *et al*., 2010). Literature is very poor of informations about the specific DMT1 –IRE and +IRE isoforms in Parkinson's Disease, these few informations appear very contrasting: while a recent study shows the down-regulation of DMT1 –IRE and the up-regulation of DMT1 +IRE (Salaza J *et al*., 2008), a genomic study failed to show DMT1 (in general, so not referred to a specific isoform) up-regulation in an M9D dopaminergic cell line treated with MPP^+ (Wang J *et al*., 2007) and another recent study using MES23.5 dopaminergic cells shows that MPP^+ treatment is sufficient to increase DMT1 -IRE expression increasing iron uptake because of a major iron infflux (Zhang S *et al*., 2009).

Different questions arised on the basis of these observations and are the object of this study:

1) I enquired if TfR2, so largely involved in iron uptake al neuronal level, coud be regulated by microRNAs;

2) I enquired about the up- or the down-regulation of DMT1 –IRE isoform in my cellular model of Parkinson's Disease;

3) I enquired about a possible microRNA Let-7d regulated expression of DMT1 –IRE isoform in the *in vitro* model used for this study.

For this, the final task was that of identify putative microRNAs which could be involed in the regulation of the expression of both DMT1 –IRE and TfR2 in an *in vitro* model of Parkinson's Disease through the following steps:

- Bioinformatics selection of microRNAs and following evaluation of their basal expression in the chosen cellular model;

- Evaluation of the effectiveness of binding between selected microRNAs and the 3'UTR from studied genes;

-MicroRNAs overexpression and evaluation of the effects on the expressions of the studied genes/proteins in the *"in vitro"* Parkinson's Diesease model.

5.1 Cellular Model: SH-SY5Y cells treated with MPP^+

SH-SY5Y

SH-SY5Y cells were chosen as cell line to reproduce an *"in vitro"* PD model, as it shows specific dopaminergic features like neurons in substantia nigra. Human Neuroblastoma Cells SH-SY5Y are a neuroblastic subclone extensively characterized by SK-N-SH (Nakagawa-Yagi *et al*., 1991; Seidenfaden *et al*., 2006), obtained in 1970 from methastatic neuroblastic tissues of a four years old female child (Biedler *et al*., 1973). Neuroblastoma is a tumor with its onset in pediatric age which originates from immature neuroblasts during the periferic nervous system develpement (Olsson *et al*., 2000). SH-SY5Y cell line is a cellular population with a simil-neuroblasts morphology, with the cell body similar to a drop (Jalava *et al*., 1990) and the cells also show short axons, growing in large part as a single layer in cell colture dishes (Biedler *et al*., 1973), while a small amount of cells, is suspended in the medium and represents cells in mithosis. These cells are a well characterized model for *in vitro* differentiation studies as a simil-neuronal phenotype that can be induced by the use of various exogen differentiating agents such as retinoic acid, nerve growth factor (NGF) and others (Seidenfaden *et al*., 2006; Peterfreund *et al*., 1997; Ammer e Schulz, 1994). Differentiation of these cells is associated with the extension of long axons and can be quantified through morphological analysis of the axon genesis (Jalava *et al*., 1990,

Seidenfaden *et al*., 2006). SH-SY5Y cell line is widely used as a model for the study of neuronal cell death caused by oxidative stress, associated with various chronic neurodegenerative diseases as Parkinson's Disease, Alzheimer's Disease and Huntington Disease (Ruffels *et al*., 2004). Scientific evidences show that the treatment of these cells with retinoic acid is not necessary when used as *in vitro* model of Parkinson's Disease. Therefore undifferentiated SH-SY5Y is more appropriate in experimental Parkinson's Disease research (Cheung YT *et al*. 2009).

5.2. MPP^+

MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, also known as "synthetic heroin") is a secondary product which is formed during the meperidine synthesis. It can lead to a reversible form of Parkinson's Disease which can be blocked by interrupting the consumption of this substance. It is also used as a fast-acting opioid analgesic drug. The MPTP alone does not have oppioid effect, but can be accidentally produced during illegal creation of MPPP (1-Methyl-4-phenyl-4-propionoxypiperidine). MPTP has side effects as its intake causes Parkinson's symptoms, since some MPPP users develops these symptoms. MPTP crosses very well the blood-brain barrier, enters glial cells where monoamino oxidase B enzymes (MAO-B) oxidate it in MPP^+ (1-methyl-4-phenylpyridine). MPP^+ enters dopaminergic cells localized in the substantia nigra, exploiting the dopamine carrier, causing

neuronal death as it inhibits mithocondrial respiration; as a consequence, oxidative stress and damage is generated along with more damage. MPTP neurotoxicity was discovered in 1976 after Barry Kidston, a 23 years old chemistry student in Maryland, synthetized and injected MPPP contaminated with MPTP; after three days he showed Parkinson's Disease symptoms. National Institute of Mental Health found traces of MPTP in his laboratory and found by accident its effects while experimenting on mice. In 1982 in seven people from Santa Clara County, California, Parkinson's Diseases was diagnosed after using MPPP contaminated with MPTP. Later, the neurologist J. William Langston, in collaboration with NIH, proved the MPTP effects, testing it on primates.

Materials and Methods

1. Bioinformatics Selection of microRNAs

PITA tool

(http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html)

is an algorithm that is used to predict miRNA targets. It is based on a parameter-free model for microRNA-target interactions that computes the differences between the free energy gainedfrom the formation of the microRNA-target duplex and the energetic cost of unpairing the target to make it accessible to the microRNA. The site accessibility is as important as the sequence match in the seed for determining the efficacy of microRNA-mediated translational repression, and the effective microRNA binding also requires the local region flanking the target to be unpaired. The energy-based scores for microRNA-target interactions, ΔΔG, predicted by the PITA tool are equal to the difference between the free energy gained by the binding of the microRNA to the target, ΔG_{duplex}, and the free energy lost by unpairing the target-site nucleotides, ΔG_{open}.

TargetScan

(http://www.targetscan.org/)

TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region

of each miRNA. As an option, nonconserved sites are also predicted. Also identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing. In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using the context + scores of the sites. As an option, predictions are also ranked by their probability of conserved targeting.

MicroCosm

(http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/)

MicroCosm Targets (formerly miRBase Targets) is a web resource developed by the Enright Lab at the EMBL-EBI containing computationally predicted targets for microRNAs across many species. The miRNA sequences are obtained from the miRBase Sequence database and most genomic sequence from EnsEMBL.

The 3'UTR of the studied genes were submitted to every previously described bioinformatics tools in order to obtain a list of microRNAs potentially binding it, helped by the score associated to microRNAs. The more the score is negative, the more the binding probability is high. Moreover, outputs from all the used tools were compared in order to select the microRNAs with the higher probability of binding.

2. Standard Cell Coltures

The K562 human myeloid leukemia cell line, the HEK293 human embryonic kidney cell line and the SH-SY5Y neuuroblastoma cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

K562 cells were maintained at 37°C with 5% CO_2 in Iscove's medium (Sigma Aldrich, Milan, Italy), supplemented with 10% fetal bovine serum (FBS) (Celbio Pero, Milan, Italy), 10 U/mL penicillin and 0.1 mg/mL streptomycin (Pen/Step) (Celbio Pero). Hek 293 cells were maintained at 37°C with 5% CO_2 in Dulbecco's Modified Eagle'sMedium (DMEM) (Sigma Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Milan, Italy), 10 U/mL penicillin and 0.1 mg/mL streptomycin (Pen/Step) (Celbio Pero). SH-SY5Y cells lines were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Milan, Italy), 10 U/mL penicillin and 0.1 mg/mL streptomycin (Pen/Step) (Celbio Pero).

3. MTS assay

SH-SY5Y cells were treated with two different concentration of MPP^+, respectively 0.5 mM and 1 mM. Cell viability after 24 hours, 48 hours and 72 hours was evaluated by an MTS assay performed by using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay System (Promega, Madison, WI, USA). It is a colorimetric method to determine

the number of viable cells in proliferation or cytotoxicity assays and contains a tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] that is reduced in presence of NADH and NADPH produced by active cells methabolism.

4. RNA extraction from SH-SY5Y cells

Cells were detached from colture dished using 1 mL of trypsin 0,05% and EDTA 0,02% solution. Subsequently cells were washed twice in a phosphate-buffered saline (PBS) (Sigma Aldrich, Milan, Italy) and lysate by 1 mL of Trizol Reagent (Invitrogen, La Jolla,CA, USA). Samples were then incubated for 5 minutes at room temperature. A volume of 200 µL of chloroform was added and the solution was mixed for 15 seconds, incubated for 2-3 minutes at room temperature and then centrifugated for 15 minutes at 12000x*g* at 4°C to obatain two phases. RNA contained in the superior white phase was precipitated by adding 500 µL of isopropyl alcohol and the solution was incubated for 10 minutes on ice. A subsequent centrifugation step (12000xg, 15 minutes, 4°C) allowed the precipitation of the RNA pellet. This pellet was washed in ethanol 75% diluited in a H_2O-diethylpyrocarbonate (DEPC) solution. Finally, RNA was resuspended in a H_2O-diethylpyrocarbonate (DEPC) solution.

5. cDNA synthesis

2 µg di RNA were retro-trancribed using iScript™ cDNA Synthesis Kits (BioRad, Milan,Italy) in order to synthesize single strand cDNAs. In a

finale volume of 20 µL were added 4µl of 5x iScript Reaction Mix, 1µl of iScript Reverse Transcriptase and Nuclease-free water till to reach the 20 µL. The complete reaction mix was incubated for 5 minutes at 25ºC followed by 30 minutes at 42ºC and a final step for 5 minutes at 85ºC.

6. TaqMan miRNAs assay

Reverse transcriptase reactions contained 40 ng RNA sample, 50 nM stem–loop reverse transcriptase primer, 1x reverse transcriptase buffer (P/N:4319981, Applied Biosystems, Branchberg, NJ, USA), 0.25 mM each of the dNTPs, 3.33 U/mL MultiScribe reverse transcriptase (P/N: 4319983, Applied Biosystems) and 0.25 U/mL RNase inhibitor (P/N: N8080119; Applied Biosystems). The 15-µL reactions were incubated in an Applied Biosystems 9700 Thermocycler for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, and then held at 4°C. Real-time (RT)-PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System. The 20-µL PCRs included 2 µL reverse transcriptase product, 10 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 mM TaqMan probe (for microRNA Let-7d, microRNA 221, microRNA-RNU6 and all the other microRNAs assayed). The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min.

7. Vector cloning (pre-microRNAs)

Pre-miR-Let-7d, pre-miR-221 and pre-miR-222 were cloned into a pcDNA3.1 vector (Promega, Madison, WI, USA) using the EcoRI–XhoI restriction sites.

Inserts preparations

All the cloned pre-miR were amplified from genomic DNA using the following primers:

Pre Let7d Clo F: CGGGAATTCCCTAGGAAGAGGTAGTA

Pre Let7d Clo R: CGGCTCGAGCCTAAGAAAGGCAGCAG

Pre 221 Clo F: AAACCTCGAGGAGAACATGTTTCCAGGTAG

Pre 221 Clo R: AAAAGAATTCTGAACATCCAGGTCTGGGGC

Pre 222 Clo F: AAACCTCGAGAGCTAGAAGATGCCATCAGA

Pre 222 Clo R: AAACGAATTCGCTGCTGGAAGGTGTAGGTA

The complete reaction mix for every amplified pre-microRNA contained 200 ng of genomic DNA, 1 µl of forward primer (10µM), 1 µl of reverse primer (10µM), 25 µl of Master Mix (Promega, Madison, WI, USA) and water to reach a final volume of 50 µl. Reactions were incubated in Veriti Thermal Cycler (Applied Biosystems, Branchberg, NJ, USA) according to the following program: an initial step at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and a final elongation step at 72°C for 7 minuts. The amplification products were visualized on 1.5% agarose gel (BioRad, Milan, Italy) and subsequently digested with restriction enzymes according to the following protocol: 3 µl Buffer 2 (10X) (New

England Biolabs), 0.8 µl EcoRI (20.000 U/ml) (New England Biolabs), 0.8 µl XhoI (20.000 U/ml) (New England Biolabs) and water to reach the final volume of 30 µl. The digestion was carried on for 4 hour at 37°C and then enzymes were inactivated at 65°C for 20 minutes.

Vector Preparation

2000 ng of pcDNA3.1 vector were digested using 3 µl Buffer 2 (10X) (New England Biolabs), 0.8 µl EcoRI (20.000 U/ml) (New England Biolabs), 0.8 µl XhoI (20.000 U/ml) (New England Biolabs) and water to reach the final volume of 30 µl. The digestion was carried on for 4 hour at 37°C and then enzymes were inactivated at 65°C for 20 minutes. Subsequently, 1000 ng of digested vector were dephosporilated at the 5' end using 3µl of NEB buffer Antarctic phosphatase (10X), 1µl of Antarctic phosphatase (New England Biolabs) and water to reach the final volume of 30 µl. The reaction was incubated at 37°C for 2 hours and then the enzyme was inactivated for 20 minutes at 65°C. The digested an dephosporilated vector, was finally purified from agarose gel (1%) (BioRad, Milan,Italy) using QuiaQuik gel extraction Kit (Quiagen).

8. Vector cloning (3'UTRs)

The full-length 3'UTR of the mRNA encoding for both DMT1 –IRE and TfR2 were cloned into the pRL-TK vector (Promega, Madison, WI, USA) downstream of the coding region of Renilla Luciferase in the XbaI site.

Inserts preparation:

The 3'-UTR of DMT1-IRE and TfR2 were amplified from genomic DNA using the following primers, containing at the 5' end the restriction site:

DMT1 –IRE Clo F: CCCTCTAGACAGCTCATTCTGGCATTCAA

DMT1 –IRE Clo R: CCCTCTAGAGCTGAAAGGGGAAAGTGATG

TfR2 Clo F: AAATCTAGACCAGTCAAGAGCTCCTCTGC

TfR2 Clo R: AAATCTAGATGGCTTATTGATATCAAGGTGGTT

The complete reaction mix for every amplified 3'UTR contained 100 ng of genomic DNA, 1 of forward primer (10 µM), 1 µl of reverse primer (10µM), 2 µl of $MgCl_2$ (25mM), 0.3 µl of Taq Gold (Roche), 5 µl of dNTP mix (2mM), 12.5 µl of 10X Buffer (Roche) and water to reach a final volume of 25 µl. Reactions were incubated in Veriti Thermal Cycler (Applied Biosystems, Branchberg, NJ, USA) according to the following program for DMT1 -IRE: an initial step at 95°C for 10 minutes followed by 35 cycles at 95°C for 30 seconds, 45°C for 40 seconds, 72°C for 3 minutes and a final elongation step at 72°C for 10 minutes. The amplification of the 3'UTR of TfR2 was performed according the following program: 95°C for 10 minutes followed by 35 cycles at 95°C for 30 seconds, 60°C for 35 seconds, 72°C for 35 seconds and a final step at 72°C for 7 minutes.

The amplification products were visualized on 1% agarose gel (BioRad, Milan, Italy) and subsequently digested with restriction enzymes according to the following protocol: 3 µl 10X Buffer 2 (New England Biolabs), 0.8 µl XbaI (20.000 U/ml) (New England Biolabs), and water

to reach the final volume of 30 µl. The digestion was carried on for 4 hours at 37°C and then enzymes were inactivated at 65°C for 20 minutes.

Vector preparation

2000 ng of pRL-TK vector (Promega, Madison, WI, USA) were digested using 3 µl Buffer 2 (10X) (New England Biolabs), 0.8 µl XbaI (20.000 U/ml) (New England Biolabs) and water to reach the final volume of 30 µl. The digestion was carried on for 4 hour at 37°C and then enzymes were inactivated at 65°C for 20 minutes. Subsequently, 1000 ng of digested vector were dephosporilated at the 5' end using 3µl of NEB buffer Antarctic phosphatase (10X), 1µl of Antarctic phosphatase (New England Biolabs) and water to reach the final volume of 30 µl. The reaction was incubated at 37°C for 2 hours and then the enzyme was inactivated for 20 minutes at 65°C. The digested an dephosporilated vector, was finally purified from agarose gel (1%) (BioRad, Milan,Italy) using QuiaQuik gel extraction Kit (Quiagen).

9. Quantitative real-time PCR

Quantitative RT-PCR (qRT-PCR) was performed using the SYBRgreen method following standard protocols with an Applied Biosystems ABI PRISM 7900HT Sequence Detection system. Relative gene expression was calculated using the $2^{(-\Delta Ct)}$ method, where Δt indicates the differences in the mean Ct between selected genes and the internal control. The β-actin genes was the internal controls for each gene. Mean

fold-change was calculated using the $2^{-(\text{average } \Delta\Delta Ct)}$ method (Livak KJ *et al.*, 2001), where ΔΔCt is the mean difference in the ΔCt between the genes and the internal control. QRT-PCR primers for each gene were designed using Primer Express software version 2.0 (Applied Biosystems). Primer sequences are the following:

DMT1 –IRE F: TGTCCTTCCTGGACTGTGGG

DMT1 –IRE R: CGTCCATGGTGTTCAGAAGATAGA

TfR2 F: GCATAATGCGGGTGGAGTTC

TfR2 R: GGCCCATGAAGATGTGGC

β-Act F: CGTGCTGCTGACCGA

β-Act R: GAAGGTCTCAAACATGATCTGGGT

The significance of the gene expression differences were determined using the Student's *t*-test; statistical significance was established at $P<0.05$. All statistical analysis was performed by Excel included in Microsoft Office 2007.

10. Western blotting

35 µg total lysates were loaded onto 12% polyacrylamide gels to evaluate the protein expression of DMT1 -IRE, onto a 10% polyacrylamide gels to evaluate the xpression of TfR2. Both gels were blotted onto polyvinylidene difluoride membranes (BioRad, Milan, Italy). The membranes were then incubated with a with a polyclonal rabbit anti-DMT1 -IRE antibody (Alpha Diagnostic) (1:500), a polyclonal rabbit anti-TfR2 antibody (Abcam) (1:500) and Anti-β-actin (Sigma) (1:1000)

was used as controls for equal loading of total lysates. The bands were quantified by densitometry to obtain optical densities (ODs) which were then normalized with respect to the normalized protein OD. We used the GS-800 Calibrated Densitometer and the Quantity One 4.6.3 program (BioRad, Milan, Italy) for densitometric analyses. The significance of protein expression differences were determined using the Student's *t*-test. Statistical significance was established at $P<0.05$.

All the antibody used to carry out all the others reported western blotting are mentioned in Table 2.

Antibody	Company - Code	Diluition
DMT1 +IRE	*Alpha Diagnostic - NRAMP 22-A*	1:500
DMT1 -IRE	*Alpha Diagnostic - NRAMP 23-A*	1:500
FTH/FTL	*Abcam - ab75973*	1:500
TfR1	*Santa Cruz - sc-32272*	1:100
TfR2	*Abcam - ab83810*	1:100
IRP2	*Santa Cruz - sc-33682*	1:500
VDAC1	*Abcam - ab119296*	1:500
b-Actin	*Sigma - A5441*	1:1000

Table 2. List of the used antibodies.

11. Site direct Mutagenesis

The site-directed mutagenesis of the miR-Let-7d binding site of DMT1 - IRE and the miR-221 binding site of TfR2 were generated according to the manufacturer's protocol of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The following primers were used for the mutagenesis (only the sense sequences are given, with the mutated nucleotides within the consensus sequence underlined and the site of deletion specified):

miR-Let-7d-Mut:

CCTAGGAACACGAATTAGATTGCATAGTTTTAG

miR-221-Mut:

CTGTGTTCGTTAGGCAACCGATCCATTGTCTGCTGGGTTTCAGG

miR-221-Del:

CTGTGTTCGTTAGGCAACA[del]ACATTGTCTGCTGGGTTTCAGGC

12. Cell transfections and luciferase assays

To evaluate the activity of miR-Let-7d on the repression of the full-length luciferase 3'-UTR constructs, the pcDNA3.1 miR-Let-7d construct (0.5 μg) and the pRL-TK 3'-UTR DMT1 -IRE target construct (0.1 μg) were cotransfected into K562 and HEL cells, using the TransFectin Lipid Reagent (BioRad, Milan, Italy) with a pGL3 CMV firefly luciferase vector for normalization. To evaluate the activity of both miR-221 and miR-222 on the repression of the full-length luciferase 3'-UTR constructs, the pcDNA3.1 miR-221 (0.5 μg) and the pcDNA3.1 miR-222 (0.5 μg) constructs and the pRL-TK 3'-UTR TfR2 target construct (0.1 μg) were cotransfected into HEK 293 cells, using the TransFectin Lipid Reagent (BioRad, Milan, Italy) with a pGL3 CMV firefly luciferase vector for normalization. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA).

13. Mithocondrial Purification

The mitochondrial extraction was performed using Mithocondrial Isolation Kit for Coltured Cells (Pierce, Rockford, IL), according to the manufacturer's procedures from treated and untreated SH-SY5Y cells.

Results

1. Evaluation of cell viability under MPP^+ treatment

Although several authors report different MPP^+ concentrations to treat SH-SY5Y to create an *in vitro* model of Parkinson's Disease, probably the most commonly used concentration is of 0,5 mM (Kim SY *et al.*, 2010).

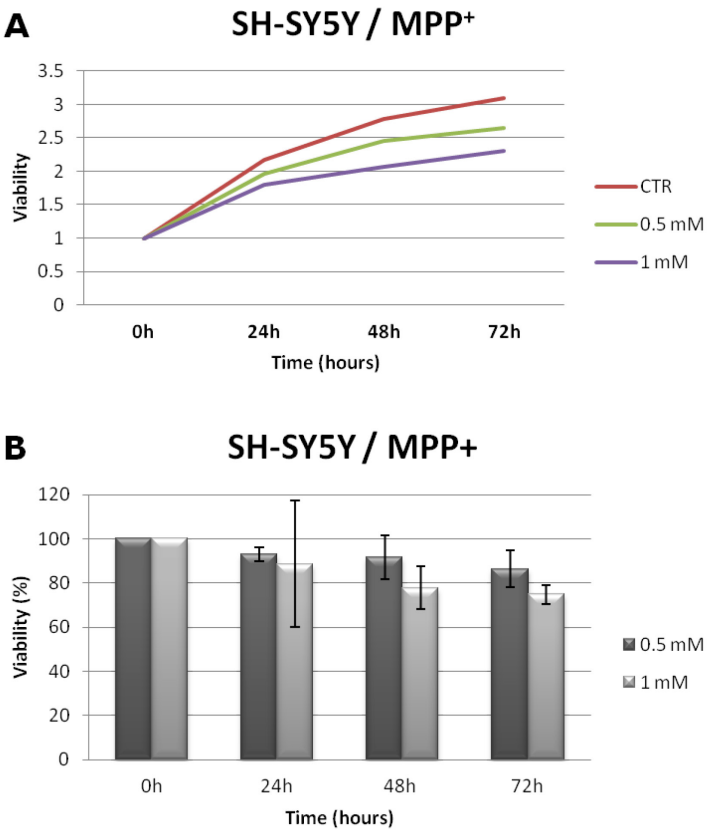

Figure 9. MTS assay on SH-SY5Y cells treated with two different concentrations of MPP^+. Cells were treated with 0.5 mM and 1 mM of MPP^+ and their vitality was evaluated after 24, 48 and 72 hours from treatment (panel A). Panel B reports data in percentage respect to the experimental point at 0 hours from treatment.

In order to evaluate the toxicity due to the use of this drug, I performed an MTS assay, testing two different concentrations of MPP^+ after 24, 48

and 72 hours. As shown in Figure 9, panel A, the MPP^+ treatment, compared with the untreated cells (CTR), reduces the viability after 24, 48 and 72 hours from treatment, although cells keep growing.

I decided to use the concentration at which cells showed a viability superior to 80% even after 72 hours from treatment. As shown in figure 9**,** panel B, there is a very little reduction in cell viability after that time, for those cells treated with the final concentration of 0.5 mM of MPP^+, while when cells were treated with a final concentration of 1 mM of MPP^+, the viability was inferior to 80% even after 24 hours. Because of these results, every other experiment performed on SH-SY5Y cells was carried out in 0.5 mM treatment condition.

2. Expression of iron metabolism protein in treated SH-SY5Y

I first analyzed the expression of the "basic" proteins involved in iron metabolism.

Accordingly to what reported in literature (Zhang *et al*., 2009) about experiments on another PD *in vitro* cell system, I found no significant alteration in the expression of DMT1 +IRE in treated cells after 24 and 48 hours from treatment, as shown in figure 10, panel A, and as confirmed by densitometric analysis shown in panel C.

No significant variations were found for FTL protein too (figure 10, A and E), while FTH (figure 10, A and D) and IRP2 (figure 10, B and G) seem to be strongly increased in treated cells.

TfR1 resulted increased in a significant way after 24 and 48 hours from treatment (figure 10, B and F).

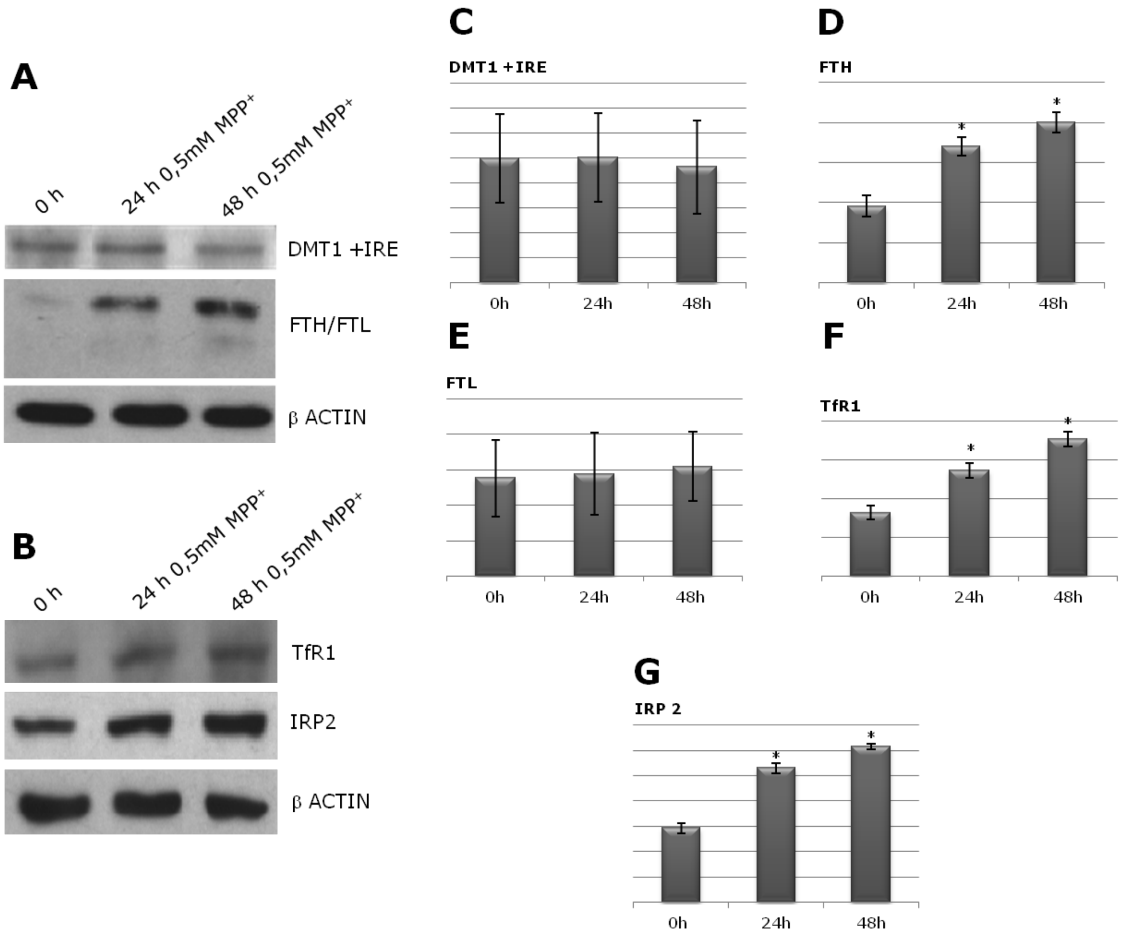

Figura 10. Protein and mRNA levels of genes involved in iron metabolism. Experiment carried out on treated and untreated cells. (A) WB for DMT1 +IRE and FTH and FTL in treated cells; (B) WB for TfR1 and IRP2 in treated cells; (C, D, E, F, G) Densitometric analysis for the blotted proteins shows a significant expression increase of FTH (D), TfR1 (F) and IRP2 (G) (*t*-test; $p<0.05$).

3. Expression of DMT1 –IRE and TfR2 in treated cells

Because DMT1 –IRE and TfR2 are the main subject of this thesis, their expression is reported in this separate paragraph. Their expression was evaluated at both mRNA and protein level by Real Time-PCR and

Western Blot. As shown in figure 11 (A) the expression of mRNA encoding for Tfr2, in treated cells, is increased if compared with the control value. Also the analysis on the protein showed the increase. Data were confirmed by densitometric evaluation of the band intensity (figure 11, B-C). The same trend was found about the expression of DMT1 –IRE: figure 11 (D) shows an over-expression of mRNA encoding for the protein. The increase was subsequently confirmed by Western Blot and densitometric analysis shown in figure 11 (E-F)

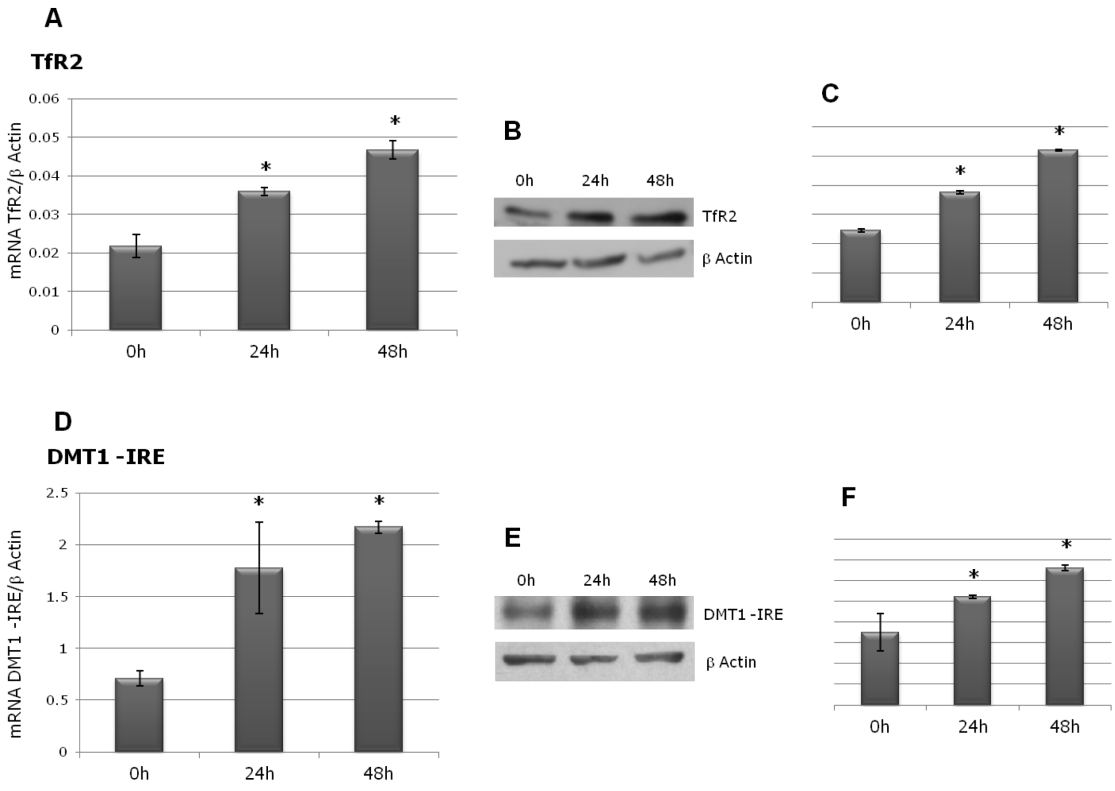

Figure 11. Expression of DMT1 –IRE and TfR2 in treated and untreated cells. Real Time-PCR shows the increase in the expression of both TfR2 and DMT1 –IRE (A, D) and the same is at protein level (B, E), confirmed by densitometric analysis (C, F). A significant increase was found for both investigated genes/proteins (*t*-test; $p<0.05$).

4. Bioinformatics selection of microRNAs potentially regulating DMT1 –IRE and TfR2

The selection by which our group identified microRNA-Let-7d as targeting DMT1 –IRE (recent published paper by Andolfo *et al*., 2010) was carried out predominantly using PITA tool on the basis of the energy-based scores for microRNA-target interactions, ΔΔG. Several prediction programs have been developed to identify potential miRNA targets (Sethupathy P *et al*., 2006). Our *in silico* analysis using the mirBase targets database (version 4) (Griffiths-Jones S, 2004) was directed towards identification of miRNAs that can potentially target the DMT1-IRE. All the analysis let us identify four potential miRNAs: miR-15a, miR-15b, miR-223 and miR-Let-7d.

For the selection of microRNAs potentially involved in the binding of the 3'UTR of TfR2, outputs from 3 different online prediction tools (MicroCosm, TargetScan and Pita tool), queried by submitting the 3'UTR of interest, were compared in order to reduce the list of microRNAs to study (I arbitrarily chose as cutoff the value -10 considering only microRNAs with a lower score). Lower is the score, higher is the probability that the microRNA is really able to interact with submitted RNA sequence. In order to reduce the number of microRNAs selected using bioinformatics methods, these results were sub-compared with literature data and particularly were selected those microRNAs expressed in general in brain derived from microarray experiment on human tissues published by Baskerville S *et al*., (2005). The list was so

reduced to about 20 different microRNAs (Table 3) ad of particular interest I obtained a short list of 5 microRNAs resulted from literature data and at list two different bioinformatics tools: microRNA-147 (PITA score = -14.24, microRNA-221 (PITA score = -18.63), microRNA-222 (PITA score = -13.08), microRNA-200c (PITA score = -11.18) and microRNA-326 (PITA score = -11.33).

microRNA	PITA score
hsa-miR-939	-24.58
hsa-miR-296-3p	-23.35
hsa-miR-637	-22.03
hsa-miR-760	-23.37
hsa-miR-423-5p	-20.98
hsa-miR-611	-18.8
hsa-miR-221	-18.63
hsa-miR-1183	-18.17
hsa-miR-1275	-17.27
hsa-miR-1292	-16.82
hsa-miR-593	-16.74
hsa-miR-574-5p	-16.37
hsa-miR-330-5p	-15.25
hsa-miR-147	-14.24
hsa-miR-222	-13.08
hsa-miR-744	-13.07
hsa-miR-608	-11.65
hsa-miR-326	-11.33
hsa-miR-200c	-11.18

Table 3. Bioinformatics selection of microRNAs potentially targeting the 3'UTR of TfR2. In red are reported all the microRNAs that resulted as the output of more then one used bioinformatics tool.

5. Expression evaluation of selected microRNAs potentially targeting the 3' UTR of the mRNA encoding for TfR2 in SH-SY5Y cells.

The basal expression of the selected microRNAs was evaluated in SH-SY5Y using Real Time-PCR carried on with stem–loop reverse transcriptase primer specific for every microRNA subjected to the assay.

The relative expression of every microRNA was obtained by the normalization on microRNA-RNU6. As shown in figure 12, almost all the microRNAs selected are very low expressed in SH-SY5Y cell line with the exception of microRNA-221 and microRNA-222 that on the contrary are very highly expressed in SH-SY5Y cells.

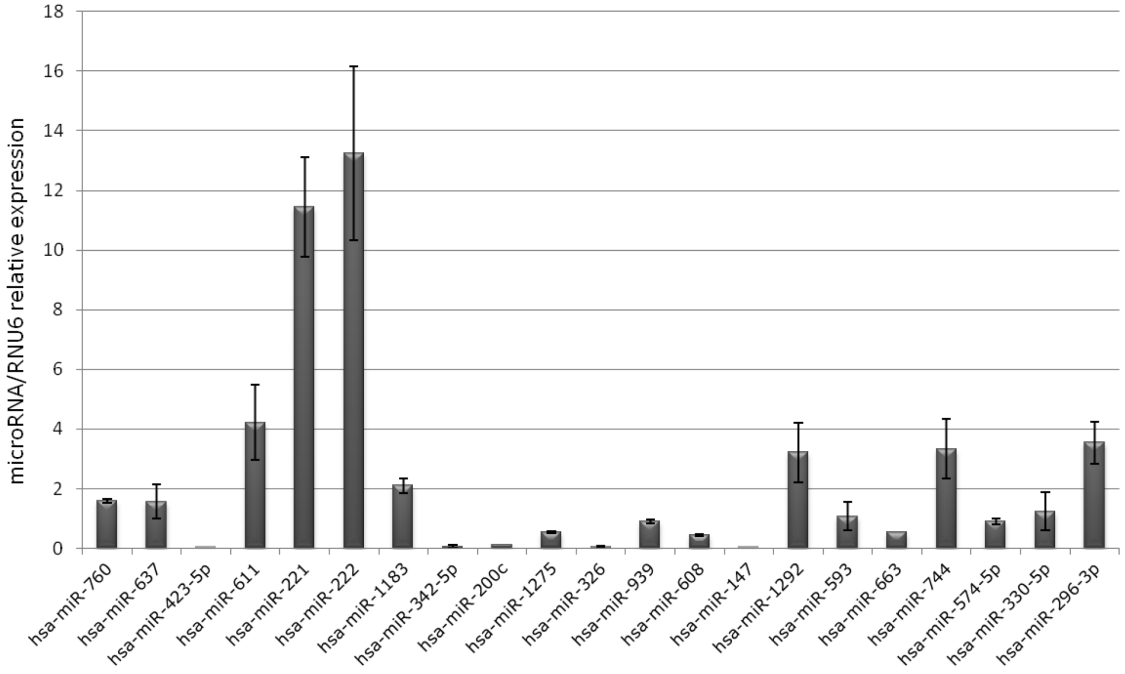

Figure 12. Relative expression of selected microRNAs in SH-SY5Y cell line. The experiment was carried out on untreated cells to evaluate the basal expression of all the selected microRNAs.

6. Expression of microRNAs in SH-SY5Y cells during the treatment with MPP^+: searching for a "TfR2 binder".

I first evaluated the expression of the 5 microRNAs (microRNA-147, microRNA-221, microRNA-222, microRNA-200c and microRNA-326) that, at the bioinformatics analysis, resulted as common result of more then one bioinformatics tools, also supported by literature data, as explained before. This experiment was performed in order to evaluate

their trend of expression during the cell treatment. The Real Time-PCR was performed on retro- trascribed microRNAs from SH-SY5Y cells treated with MPP^+ and the relative expression was evaluated normalizing on the expression of microRNA-RNU6 endogenous control. Figure 13 (A) shows the relative basal expression of the five sub-selected microRNAs in SH-SY5Y cell.

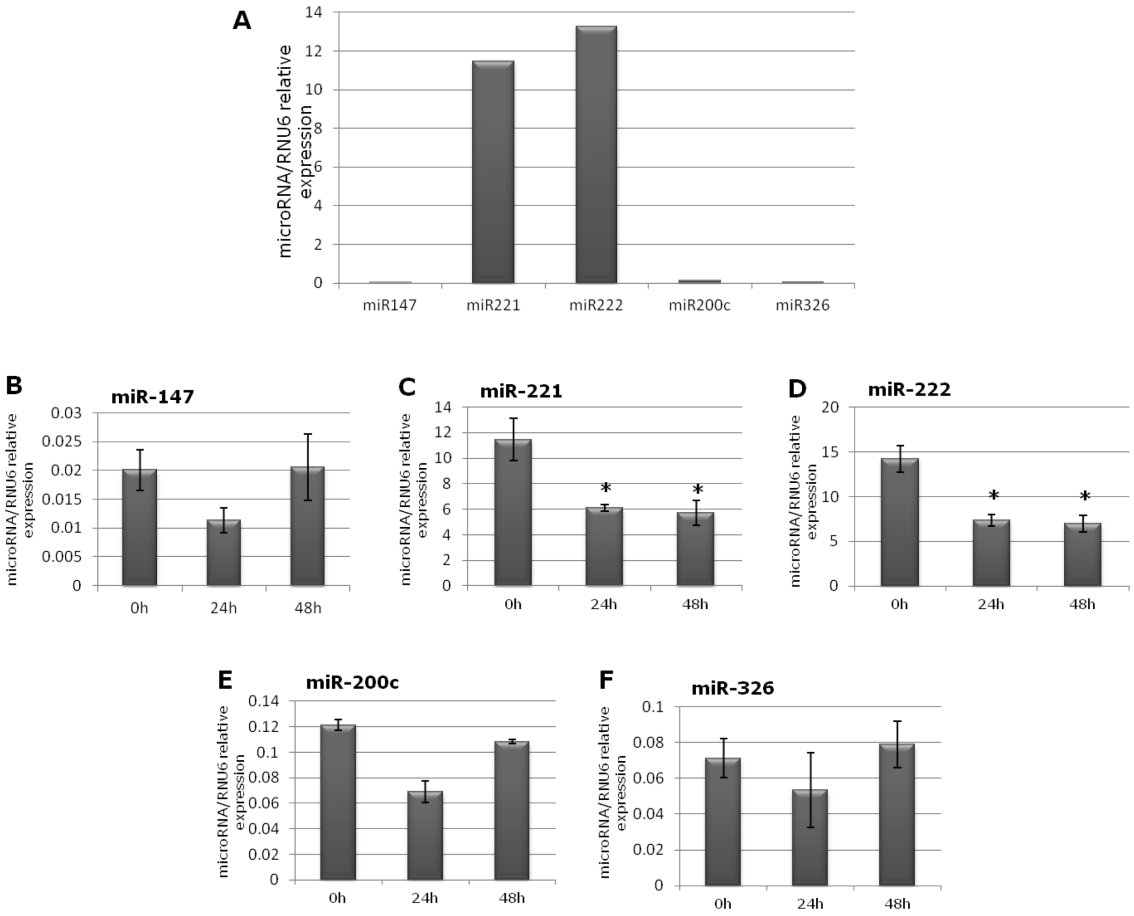

Figure 13. Sub-selected microRNAs expression in SH-SY5Y cells during the treatment. (A) miR-147, miR-200c and miR-326 resulted very low expressed in untreated SH-SY5Y cells. miR-221 and miR-222 were highly expressed in the same cells. (B, E, F) miR-147, miR-200c and miR-326 showed not consistent variations in their expression after 24 and 48 hours from treatment while both miR-221 and miR-222 resulted significantly decreased after 24 and 48 hours from treatment (C, D) (*t*-test; $p<0.05$).

As shown, the expression of both microRNA-221 and microRNA-222 is very high when compared with the other studied microRNAs. In fact, the expression of microRNA-147, microRNA-200c and microRNA-326 is very low and quite near the zero in SH-SY5Y cell line.

Anyway I decided to evaluate to analyze the expression of all the five sub selected microRNAs during the the treatment, after 24 and 48 hours to better understand if the MPP^+ treatment could induce variations in their expression levels. Moreover, I was very confident in microRNA-147 because it was the only one microRNA, predicted with a good score as the potential microRNA targeting TfR2 by all the three queried bioinformatics tools. The relative expression of the mentioned microRNAs is reported in figure 13 (panels B-F). MicroRNAs -147, -200c and -326 showed a no specific trend during the treatment and moreover the low reduction observable after 24 hour from treatment with MPP^+ results not significant (*t*-test) (figure 13, B, E and F).

On the contrary, the expression levels in SH-SY5Y cells for microRNA-221 and microRNA-222 show a trend in reduction after 24 and 48 hours from MPP^+ treatment. The reduction resulted significant already after 24 hours from treatment (figure 13, C and D) and it is maintained after 48 hours. Being TfR2 over-expressed in SH-SY5Y cells treated with MPP^+ at both mRNA and protein level (figure 11, A), I was searching for a microRNA potentially targeting it, so showing an opposite trend (figure 14). For these reasons, the best candidate for the subsequent studies were microRNA-221 and microRNA-222.

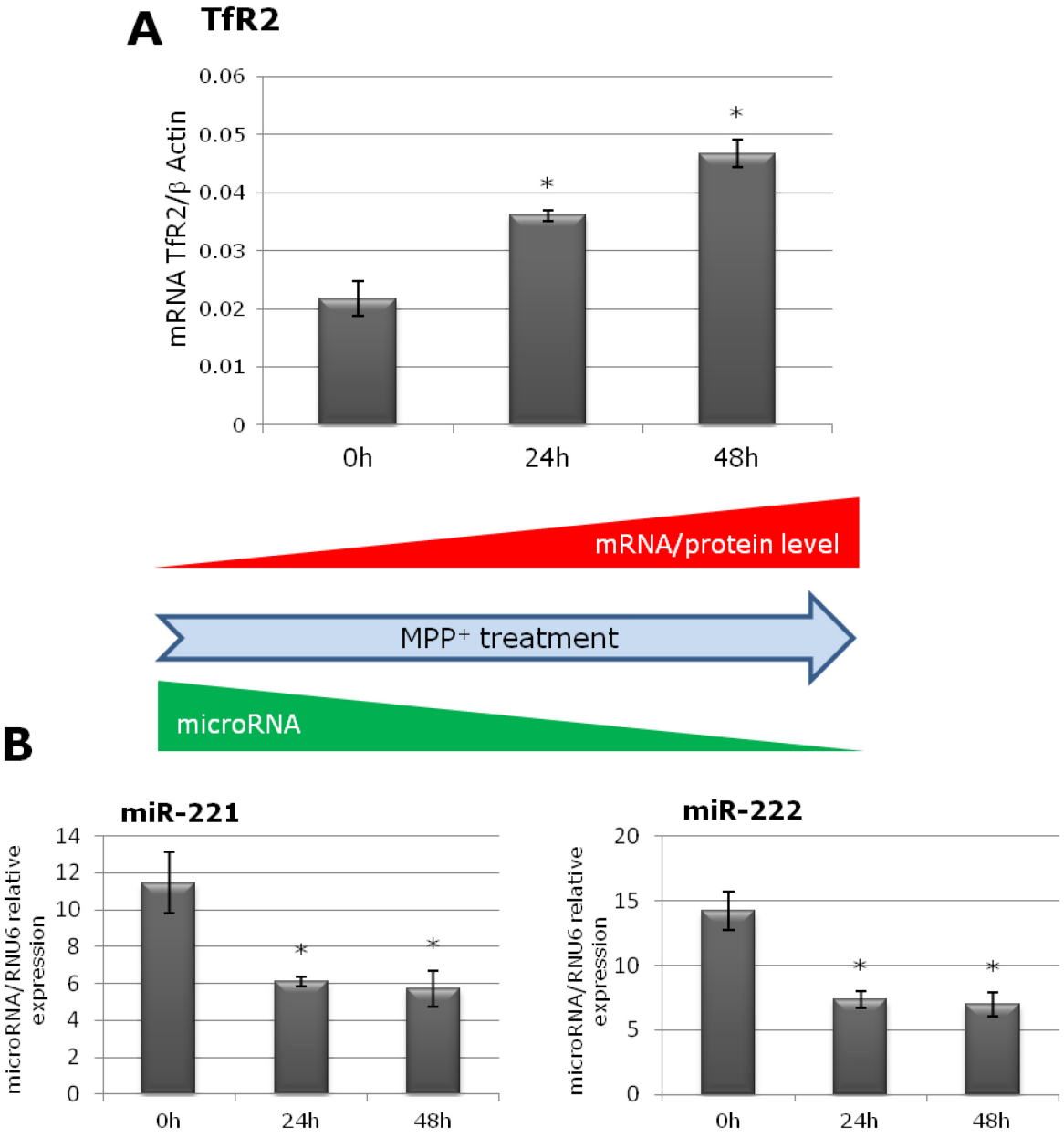

Figure 14. Opposite trend of expression between TfR2 and microRNA-221/microRNA-222. This trend suggests a potential expression regulation of TfR2 mediated by microRNA-221 and microRNA-222. Panel A shows the TfR2 mRNA increase after 24 and 48 hours from treatment while panel B shows the microRNAs reduction in the same experimental points.

7. Expression of microRNA-Let-7d in SH-SY5Y cells during the treatment with MPP^+.

As explained before, the activity of microRNA-Let-7d in the expression regulation on DMT1 –IRE was recently published by our group (Andolfo *et al*., 2010). Being DMT1 –IRE up-regulated during the treatment with MPP^+ in my PD *in vitro* model (figure 11,D-F), I enquired if the same regulation mechanism could be active in this different cell system. The first step to answer this question was the evaluation of the microRNA-Let-7d expression in SH-SH5Y cells and, moreover, if it resulted altered in its expression in SH-SY5Y cells treated with MPP^+.

The Real Time-PCR was performed on retro-transcribed microRNA-Let-7d from SH-SY5Y cells treated with MPP^+ and the relative expression was evaluated normalizing on the expression of microRNA-RNU6 endogenous control. As shown in figure 15 (B), microRNA-Let-7d levels resulted strongly and significantly reduced in treated cells after 24 and 48 hours from treatment. Also in this case is possible to observe an opposite trend in the expression of DMT1 –IRE (figure 15, A) respect to the expression of the considered microRNA.

This data suggest not only the fact that microRNA-Let-7d is expressed in SH-SY5Y cells but also suggest it potential effect on the regulation of DMT1 –IRE in my *in vitro* PD model.

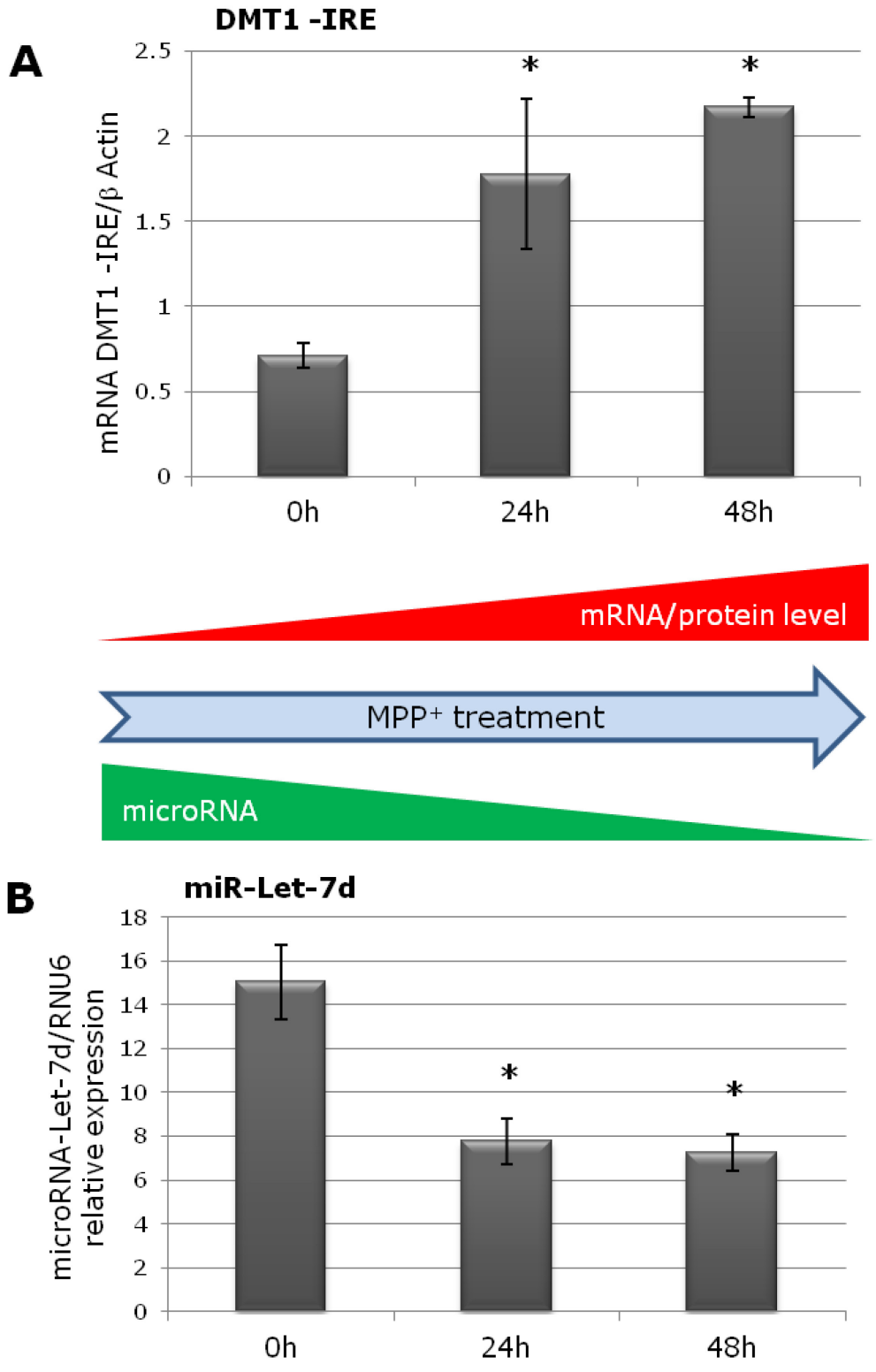

Figure 15. Opposite trend of expression between DMT1 -IRE and microRNA-Let-7d. This trend suggests a potential expression regulation of DMT1-IRE mediated by microRNA-Let-7d. Panel A shows the DMT1 -IRE mRNA increase after 24 and 48 hours from treatment while panel B shows the microRNA reduction in the same experimental points.

8. Evaluation of the binding between microRNA-221 and microRNA-222 and the 3'UTR of mRNA encoding for TfR2 and the binding between microRNA-Let-7d and the 3'UTR of the mRNA encoding for DMT1 –IRE

To verify the bioinformatics predictions, a renilla luciferase reporter vector was constructed that contained the full-length 3'-UTR of human DMT1-IRE mRNA, the expression of which was driven by the thymidine kinase (Tk) promoter (Tk-ren/DMT1-IRE). Pre-miR-Let- 7d was cloned into a mammalian expressing vector (pcDNA3.1). The reporter construct (Tk-ren/DMT1-IRE) and miR-Let-7d were co-transfected in K562 cells, with the pGL3-CMV-firefly luciferase vector to normalize for transfection efficiency.

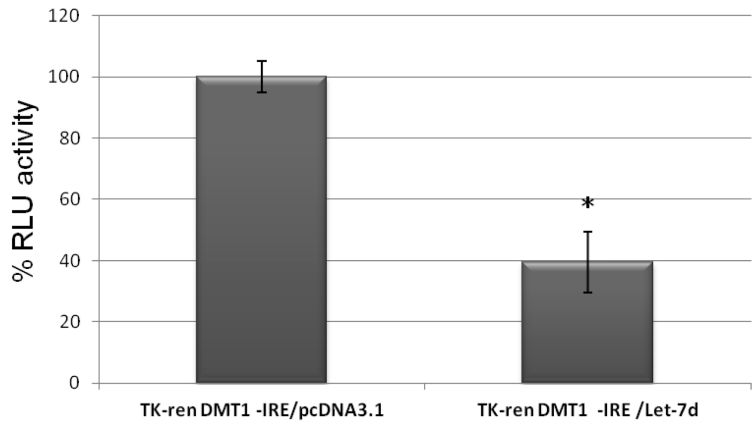

Figure 16. Luciferase assay to evaluate the binding between DMT1 –IRE 3'UTR and microRNA-Let-7d. K562 cells transfected with the construct expressing microRNA-Let-7d showed a significant reduction of the luciferase activity (*t*-test; $p<0.05$).

Interestingly, the relative luciferase activity was markedly decreased (about -70%) in cells co-transfected with the Tk-ren/DMT1-IRE

construct and miR-Let-7d ($P<0.05$) which indicates miR-Let-7d binding to the 3'-UTR of the DMT1-IRE, resulting in decreasing luciferase protein expression (Figure 16).

A similar experiment was planned to test the binding between microRNA-221 and microRNA-222 with the 3'UTR of the mRNA encoding for TfR2. A renilla luciferase reporter vector was constructed, containing the full-length 3'-UTR of human TfR2 mRNA, the expression of which was driven by the thymidine kinase (Tk) promoter (Tk-ren/3'UTR TfR2). Pre-miR-Let-221 and pre-miR-222 were cloned into a mammalian expressing vector (pcDNA3.1). The reporter construct (Tk-ren/3' UTR TfR2) and pre-miR-221 were co-transfected in HEK293 cells, with the pGL3-CMV-firefly luciferase vector to normalize for transfection efficiency. The same co-transfection (using Tk-ren/3' UTR TfR2 and the construct containing pre-miR-222) was carried out to test also the ability binding the studied 3'UTR by pre-miR-222.

As shown in figure 17 (A), the relative luciferase activity was strongly decreased (-70%) in cells co-transfected with the Tk-ren/3'UTR TfR2 construct and miR-221 ($P<0.05$) which indicates miR-221 is really able to bind the 3'-UTR of the mRNA encoding for TfR2, resulting in a decreased luciferase protein expression.

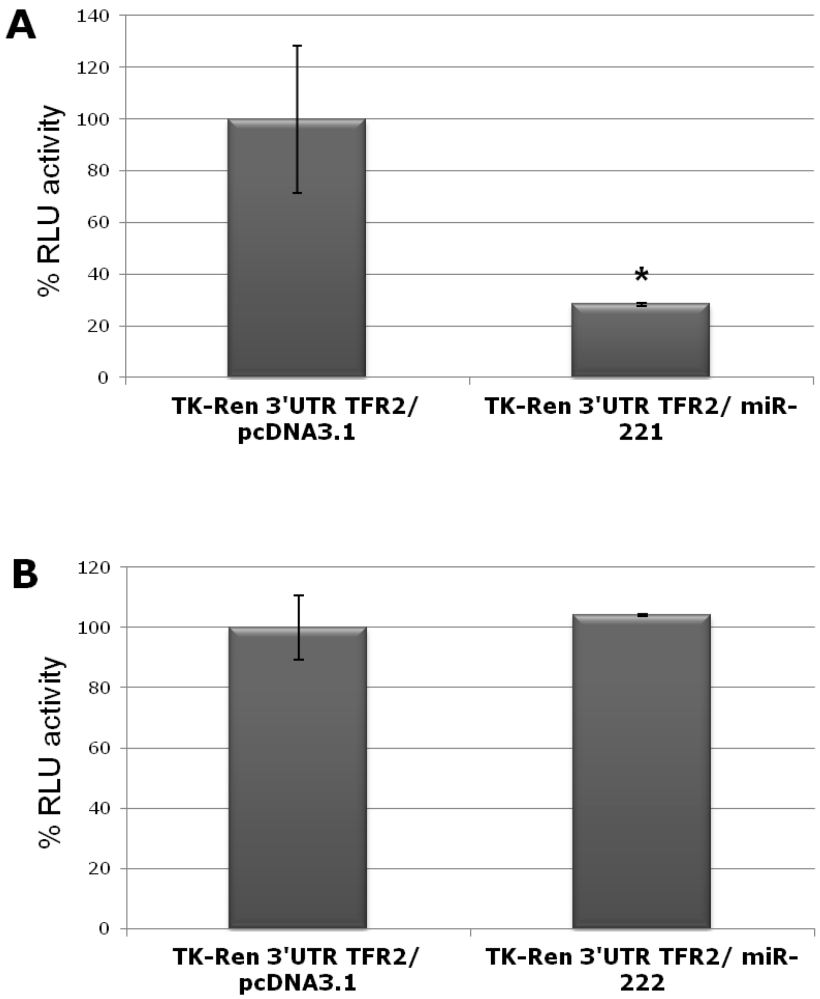

Figure 17. Luciferase assay to evaluate the binding between TfR2 3'UTR and microRNA-221/microRNA-222. (A) HEK293 cells transfected with the construct expressing microRNA-221 showed a significant reduction of the luciferase activity (*t*-test; $p<0.05$). (B) HEK293 cells transfected with the construct expressing microRNA-222 showed no alterations of the luciferase signal, suggesting that microRNA-222 is not able to bind the 3'UTR of TfR2.

As concerns the luciferase assay referred to the study of microRNA-222, since the luciferase signal in those cells transfected with the construct containing the microRNA is comparable to the luciferase signal of those cells transfected with the control vector, this suggests that microRNA-222 is not able to bind the 3'UTR of the mRNA encoding for TfR2 cloned at the 3' end of the renilla luciferase gene. The lack of this binding

prevent the regulation of renilla luciferase expression and the final effect is the same production of the protein figure 17 (B).

This data allowed me to exclude microRNA-222 from the subsequent studies since it is not a potential "regulator" of TfR2. On the contrary, microRNA-221 could be involved in such regulation.

9. Mutagenesis of microRNA-let-7d and microRNA-221

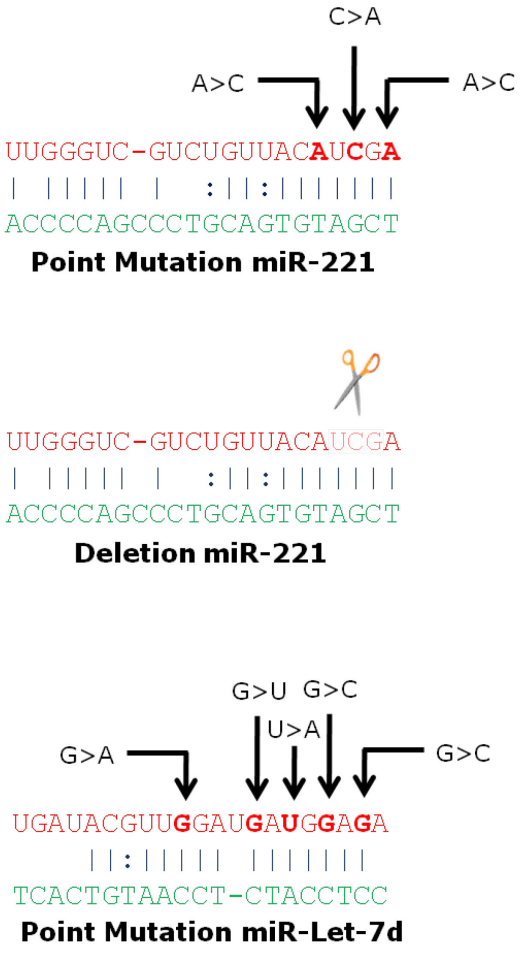

Figure 18. Mutagenesis design. Three point mutation (shown in bolt) were introduced in pre-microRNA-221 cloned into the pcDNA3.1 expression vector and in the other construct were removed three bases (reported in transparency). In pre-microRNA-Let-7d cloned in the same vector, five point mutations were introduced (shown in bolt).

MicroRNA-221 cloned into the pcDNA3.1 vector was mutagenized by introducing point mutations and deletion in the region that better binds the 3'UTR of the mRNA encoding for TfR2, localized in its 5'UTR. A first construct was created introducing three point mutations in alternating positions while the deleted one was created removing three consecutive bases as shown in figure 18. MicroRNA-Let-7d was mutagenized introducing 5 poin mutations in the region that better matchs with the 3'UTR of DMT1 –IRE as shown in figure 18 (bottom).

10. Mutagenized microRNAs restore the luciferase activity in transfected cells.

Luciferase assay was repeated in K562 cells tranfected with wild-type (WT) and mutated microRNA-Let-7d. Also in this case when cells were transfected with the WT microRNA, le luciferase activity resulted decreased. On the contrary, when cells were transfected with the mutated microRNA, the luciferase signal was restored (figure 19). These data confirm that microRNA-Let-7d is really able to bind the DMT1 –IRE 3'UTR repressing luciferase expression. In fact, when the cells are transfected, the expressed mutated microRNA is no more able to bind the co-transfected vector containing the 3'UTR, and the lack of the binding inhibits the degradation of the mRNA encoding for the luciferase, restoring the complete signal (figure 19).

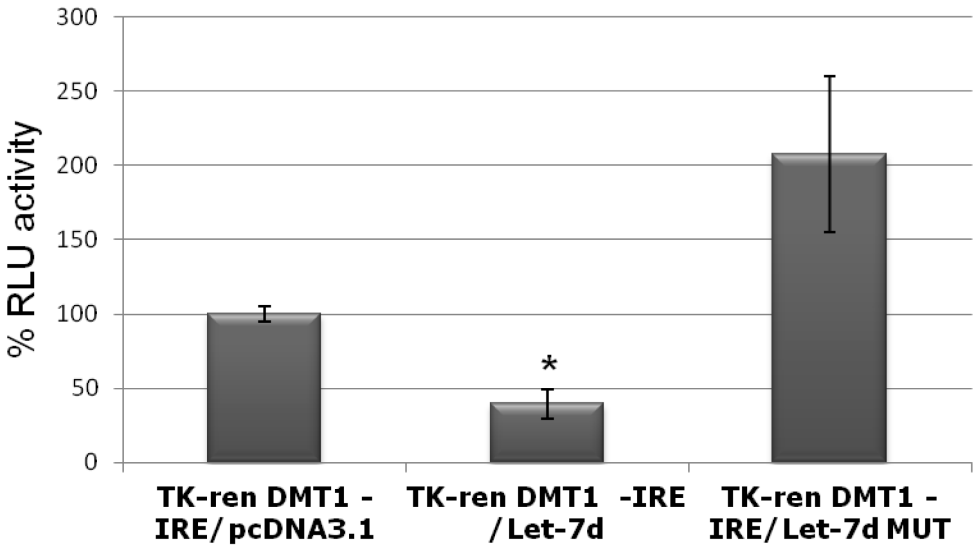

Figure 19. Luciferase assay with the mutagenized microRNA-Let-7d. K562 cells transfected with the mutated form of microRNA-Let-7d showed a complete restore of the luciferase signal when compared with cells transfected with the WT form of microRNA-Let-7d (*t*-test; $p<0.05$).

In regards to the study of microRNA-221 and the 3'UTR of TfR2, the repeated luciferase assay, also in this case shows that when HEK293 cells were transfected with the WT microRNA, the luciferase activity resulted decreased. On the contrary, when cells were transfected with the mutated microRNA, both for point mutations and deletion, the luciferase signal was restored (figure 20). The transfection with WT construct gave a significant reduction again. Both constructs, containing the point mutated and deleted forms of microRNA-221 loss the ability to bind the 3'UTR of the mRNA encoding for TfR2 and this causes their inability to negatively "regulate" the protein expression, so the

luciferase activity was reinstated confirming the potential regulation activity due to microRNA-221.

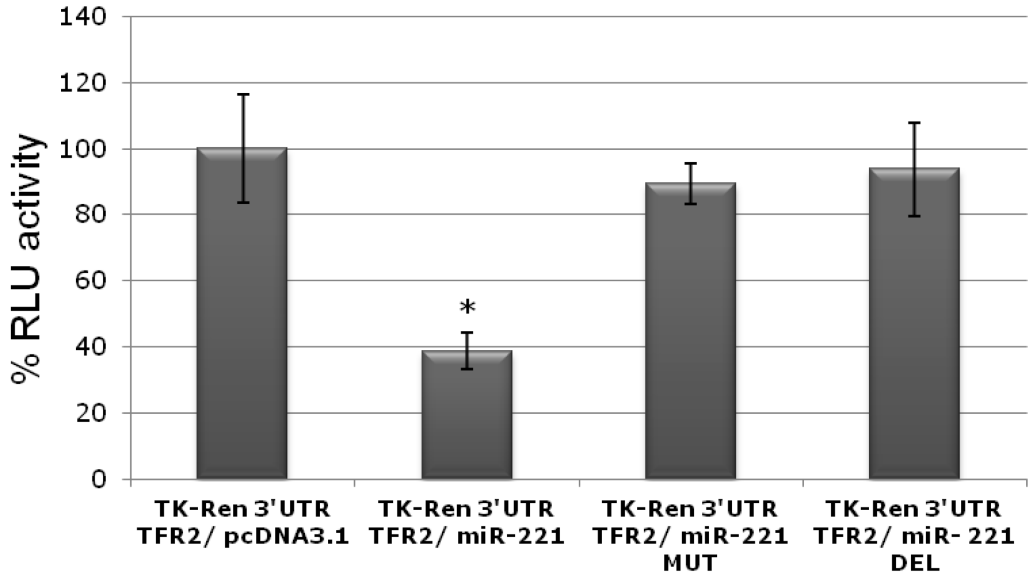

Figure 20. Luciferase assay with the mutagenized microRNA-221. HEK293 cells transfected with both the mutated forms of microRNA-221 showed a complete restore of the luciferase signal when compared with cells transfected with the WT form of microRNA-221 (*t*-test; $p<0.05$).

11. Activity of microRNA-Let-7d and microRNA-221 on endogenous targets.

To evaluate if the studied microRNAs were able to function on their respectively endogenous targets, they were transfected in SH-SY5Y cells treated with MPP^+ and their targets were evaluated at both mRNA and protein level. In fact, as shown in figure 21**,** when cells are tranfected with the wild type microRNA-Let-7d, this results in a reduction in the expression of both mRNA encoding for DMT1 –IRE and the same is analyzing the protein expression.

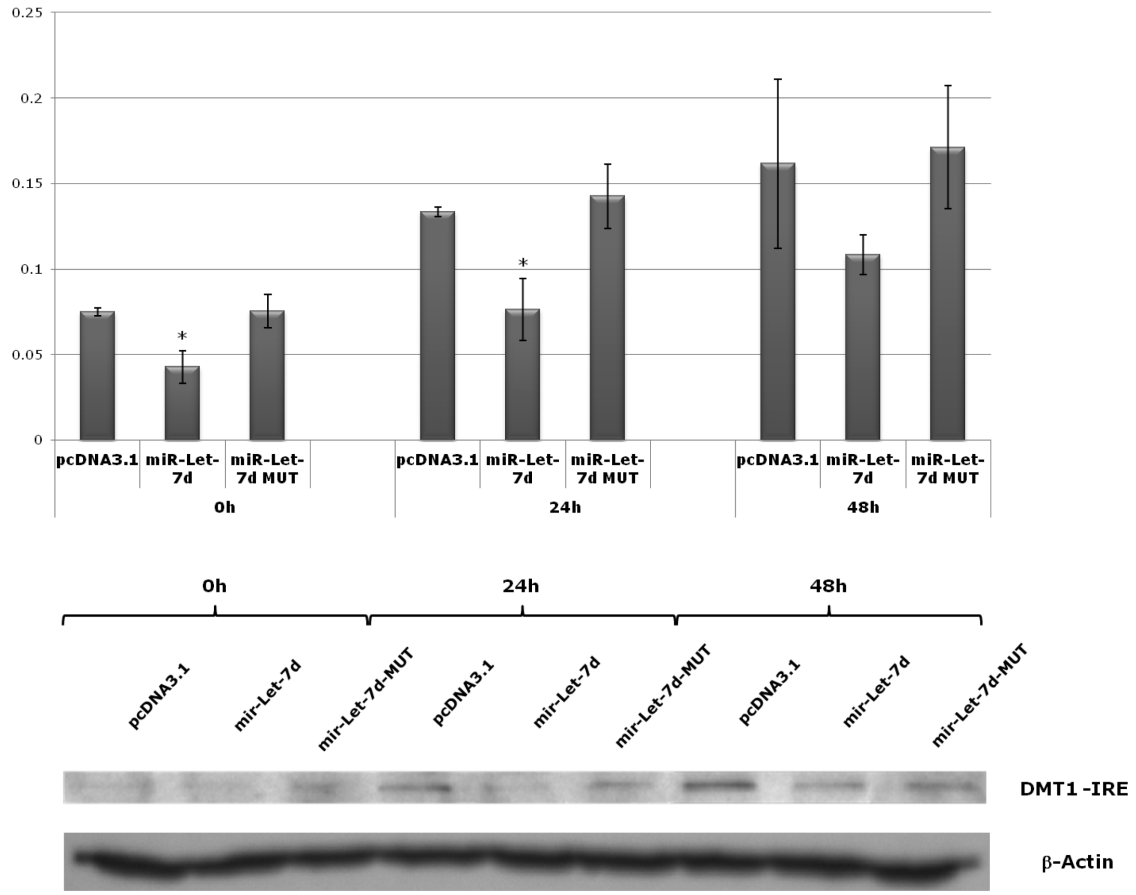

Figure 21. : Expression of DMT1 –IRE in transiently transfected cells with WT and mutated microRNA-Let-7d after 0, 24 and 48 hours from treatment with MPP^+. In the upper part of the image is reported the mRNA expression level. The increasing trend in the expression of DMT1 –IRE is maintained after 24 and 48 hours from treatment but in those experimental points in which an over-expression of microRNA-Let-7d was induced, the mRNA level decreased if compared with control cells and with cells transfected with the mutated microRNA (*t*-test; $p<0.05$) and the same happened at protein level (WB in the bottom).

Even in this case, the up-regulated trend in DMT -1 expression after 0, 24 and 48 hours from treatment with MPP^+ is maintained and when cells are transfected with the mutated construct, the ability of microRNA-Let-7d to bind the 3'UTR of DMT1 –IRE is lost and this causes the lack of the regulation of DMT1 –IRE expression and there is the

restore in the production of mRNA encoding for DMT1 –IRE and its corresponding protein.

Concerning to the experiment performed on the same cell system to evaluate the ability of microRNA-221 to reduce the expression of TfR2, also in this case, the cells transiently transfected with the construct in which the wild type microRNA was cloned, showed a reduction in both mRNA encoding for TfR2 and in the corresponding protein too.

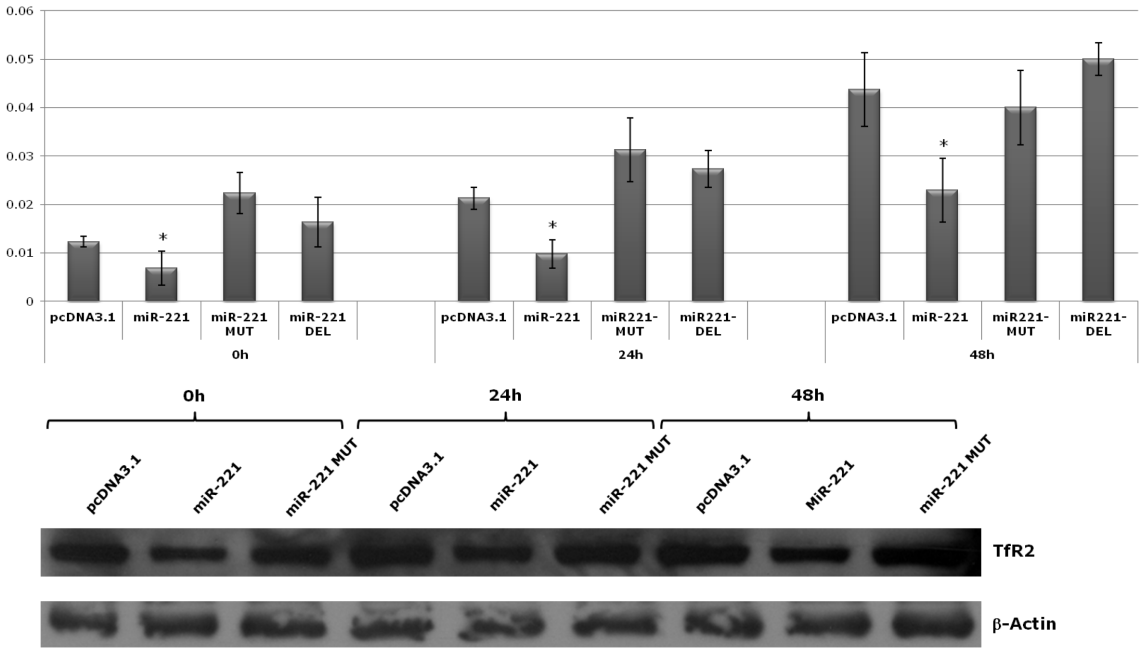

Figure 22. Expression of TfR2 in transiently transfected cells with WT and mutated microRNA-221 after 0, 24 and 48 hours from treatment with MPP^+. In the upper part of the image is reported the mRNA expression level. The increasing trend in the expression of TfR2 is maintained after 24 and 48 hours from treatment but in those experimental points in which an over-expression of microRNA-221 was induced, the mRNA level decreased if compared with control cells and with cells transfected with the mutated microRNAs (*t*-test; $p<0.05$) and the same happened at protein level (WB in the bottom). Only the construct containing point mutations in pre-microRNA-221 was tested in the WB experiment.

Even in this case the up-regulation trend in the expression of TfR2 is conserved after 0, 24 and 48 hours from treatment with the drug and when cells were transfected with both mutated and deleted microRNA-221, the lack in the binding with the 3'UTR of the mRNA encoding for TfR2 causes the restore in the production of TfR2.

Because luciferase assay (figure 20) and Real Time-PCR on SH-SY5Y treated cells (figure 22, upper part) showed that both the mutated constructs were able to work and both losed the ability to bind the target 3'UTR, only the one containing point mutations was tested for Western Blot analysis.

According to these preliminary data, both microRNA-Let-7d and microRNA-221 are really able to regulate the expression of the respectively genes/proteins involved in iron metabolism, DMT1 –IRE and TfR2 by binding the 3'UTR of their mRNA. Probably their activity mediates the degradation of the mRNAs, since the effect of the microRNAs are already observed at mRNA level and subsequently at protein level.

12. Mithocondrial expression of TfR2 in SH-SY5Y cells treated with MPP^+

Mithocondria from SH-SY5Y cells treated with MPP^+ at 0, 24 and 48 hours were purified in order to evaluate the expression of TfR2 on mithocondrial membranes. On the contrary of what reported in

literature (Mastroberardino *et al*., 2009), as shown in figure 23, I found no over-expression of TfR2 in mithocondria but instead a not significant reduction is slightly detectable. Moreover, a perfect purification of the above mentioned organelles is very difficult to obtain in laboratory. This supports the idea that probably that result is due to potential contaminations of the used samples and that probably TfR2 is completely absent on mithocondrial cell membranes.

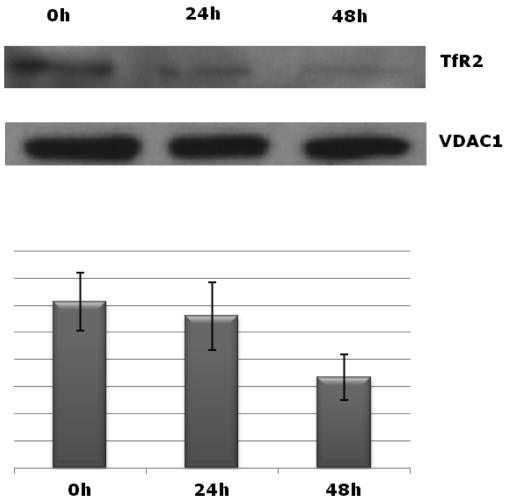

Figure 23. Mithocondrial expression of TfR2 in SH-SY5Y cells treated with MPP^+. Although is seems there is a consistent reduction in the mithocondrial expression of TfR2 after 48 hours from treatment, it resulted not significant at the *t*-test, suggesting no alteration in protein expression during the treatment.

Discussion

Dopaminergic cell death in the substantia nigra is central to Parkinson's disease (PD) but the neurodegenerative mechanisms have not been completely elucidated. The normal substantia nigra has a higher concentration of iron than the liver, which is the body's main iron store (Gotz ME *et al*., 2004). Iron accumulation in dopaminergic neurons and glial cells in the substantia nigra of PD patients may contribute to the generation of oxidative stress, protein aggregation and neuronal death. However, the mechanisms involved in iron accumulation remains unclear. Moreover, literature is very rich in contradictions about iron metabolism in Parkinson's Disease and this make the study of PD very complex. For example, a recent study shows the down-regulation of DMT1 –IRE and the up-regulation of DMT1 +IRE (Salaza J *et al*., 2008), a genomic study failed to show DMT1 (in general, so not referred to a specific isoform) up-regulation in an M9D dopaminergic cell line treated with MPP^+ (Wang J *et al*., 2007) and another recent study using MES23.5 dopaminergic cells shows that MPP^+ treatment is sufficient to increase DMT1 -IRE expression increasing iron uptake because of a major iron influx (Zhang S *et al*., 2009). About TfR2 in Parkinson's Disease literature shows even less information. In fact, the first demonstration that TfR2 is up-regulated in PD dates back to 2009 when Mastroberardino *et al*. (2009) not only found an increase of TfR2 in

dopaminergic neurons in PD but even suggests a novel transferrin/TfR2-mediated mitochondrial iron transport system disrupted in Parkinson's Disease proposing TfR2 as directly involved in the mithocondrial iron uptake because of the presence of a particular aminoacidic sequence able to target the protein in mithocondrial membranes.

Tightly regulated iron homeostasis is essential for maintenance of life in eukaryotic organisms. Stable plasma concentrations and adequate levels of cellular iron are maintained through a balance of multiple processes that regulate its absorption, transport, storage and recycling (Andrews NC, 2008; De Domenico I *et al*., 2008). DMT1 has a crucial role in this iron homeostasis because it is an H^+/divalent metal symporter that is involved in intestinal non-heme iron uptake, as well as in the peripheral iron cycle, as seen in erythroblasts (Iolascon A *et al*., 2009; Gruenheid S *et al*., 1995; Gunshin H *et al*., 1997). DMT1 expression is finely regulated by different mechanisms. One involves the iron-responsive element (+IRE) in the 3'-UTR of the DMT1 +IRE which can alter mRNA stability according to iron status (Tabuchi M *et al*., 2002; Galy B *et al*., 2008; Gunshin H *et al*., 1997). The other isoform, DMT1 –IRE is characterized by the absence of the element (+IRE) in its mRNA 3'UTR so the regulation of its expression is not modulated by the iron concentration into the cells and it is not mediated by Iron Regulative Proteins (IRPs). For these reasons other regulative mechanisms are hypothesized. We recently suggested a novel mechanism of regulation that involves microRNA-Let-7d and we proved

that in erythroid cells. Particularly, we found that that in erythroid cells, DMT1-IRE expression is under the regulation of microRNA-Let-7d. DMT1-IRE and miR-Let-7d are inversely correlated with CD34(+) cells, K562 and HEL cells during erythroid differentiation. Moreover, over-expression of miR-Let-7d decreases the expression of DMT1-IRE at the mRNA and protein levels in K562 and HEL cells. MiR-Let-7d impairs erythroid differentiation of K562 cells by accumulation of iron in the endosomes (Andolfo I *et al*., 2010). As explained in another section, microRNAs are now well recognized as important post-transcriptional regulators of gene expression. Their mechanisms of action have been intensively studied more recently, and their effects are known to be exerted primarily through suppression of mRNA translation or by promotion of the physical destruction of mRNA after the microRNA hybridizes with the 3'-UTR of their mRNA target(s). The first purpose of this study was to validate the activity of microRNA-Let-7d in the regulation of DMT –IRE in an *in vitro* model of Parkisnon's Disease. Here I showed that DMT1 –IRE is up-regulated in SH-SY5Y cells treated with MPP^+. This trend of expression is opposite to that of miR-Let-7d, a putative regulator of DMT1-IRE. In this context, I first examined whether miR-Let-7d can mediate the repression of a luciferase/DMT1-IRE 3'-UTR reporter construct in K562. Interestingly, miR-Let-7d produced a 60% decrease in the luciferase activity, indicating that it binds to the 3'-UTR of the DMT1-IRE mRNA and decreases the luciferase protein translation. Then I evaluated the regulation of DMT1 -IRE after

over-expression of miR-Let-7d. Here, I have shown that the endogenous DMT1-IRE is down-regulated by miR-Let-7d in my *in vitro* model of Pakinson's Disease, at the mRNA levels, as previously described for other members of the miR-Let-7 family (Roush S *et al.*,2008), and subsequently at protein levels. When cells were transiently transfected with the mutated microRNA-Let-7d, this cause the lack of the binding between the microRNA and its target and the DMT1 –IRE endogenous expression was restored at a level comparable to the control one suggesting that microRNA-Let-7d is no more able to regulate DMT1 –IRE expression.

A similar remark is for Transferrin Receptor type 2. Transferrin receptors are carrier proteins for transferrin. They are needed for the import of iron into the cells and import iron by internalizing the transferrin-iron complex through receptor-mediated endocytosis. TfR2 shares about 45% homology with TfR1, but its function in iron homeostasis remains elusive (Kawabata *et al.*, 1999). As reported before, differently for what happens in the regulation of the quite ubiquitously expressed Transferrin Receptor type 1 (TfR1), TfR2 can not be regulated by IRPs because of the lack on the IRE elements at the 3'UTR of its mRNA. For this reason, also in this case a different regulation mechanism can be supposed. The second aim of this study was firstly to verify the potential activity of microRNA-221 to function as a "TfR2 regulator" and then to verify its regulation in Parkinson's Disease using SH-SY5Y cell line treated with MPP^+.

Here I showed that TfR2 is up-regulated in SH-SY5Y cells treated with MPP^+. This trend of expression is the opposite to that of microRNA-221, the microRNA that I a selected as a putative regulator of TfR2. In this context, I first examined whether microRNA-221 can mediate the repression of a luciferase/TfR2 3'-UTR reporter construct in HEK293. Interestingly, miR-221 produced a 70% decrease in the luciferase activity, indicating that it binds to the 3'-UTR of the TfR2 mRNA and decreases the luciferase protein translation. Then I evaluated the regulation of TfR2 after over-expression of microRNA 221. Here, I have shown that the endogenous TfR2 is down-regulated by miR-Let-7d in the studied *in vitro* model of Pakinson's Disease, at both mRNA level and protein level.

Another unanswered question is if the iron-overload in PD is to be ascribed to DMT-1 or TfRs. Also in this case unclear literature data are reported as discussed elsewhere. For example, while Mastroberardino *et al*. (2009) tries to attribute the iron excess in dopaminergic neurons in PD to TfR2, Hirsch (2009) attributes the iron overload as due to the excluded a role of transferrin and its receptor in iron accumulation.

According to what I found in my cellular model, a synergic action of both TfR2 and DMT1 –IRE can be suggested. Both proteins resulted increased in SH-SY5Y cells and no contradiction can be seen in these findings following this proposed model: TfR2 increases and it is involved in iron cellular uptake. The fact that I found no TfR2 increase on mithocondrial surface (in contrast with what reported by

Mastroberardino *et al*., 2009) suggests that probably the over-expression is on cell membrane, and this could justify the excess of iron influx. The complex Tf/TfR2 let the iron enter the cell by endocytosis (figure 24). It is well known that the subsequent acidification of the endosome causes the release of iron from Transferrin and it is also well known that DMT1 –IRE is the direct liable protein involved in the transport of iron from the endosome to the inner cellular compartment (as widely discussed in the introduction session). Here, I also found the over-expression of DMT1 -IRE and this is consistent with the presence of a great quantity of iron into the cell (figure 24). Probably the up-regulation of only one of the studied proteins, would not be sufficient to justify the excess of iron influx. It would be expected that, under condition of cellular iron overload, iron import machinery would be post-transcriptionally regulated. As shown here, likely the regulation of both DMT1 –IRE and TfR2 is mediated by microRNAs (respectively microRNA-Let-7d and microRNA-221). The activity of the studied microRNAs is carried out in terms of mRNA degradation rather than in blocking of the translation since the reduction of the expression is already found at mRNA levels and confirmed at protein levels.

As explained, DMT1 –IRE and TfR2 expressions are not regulated by intracellular iron levels and their levels remain elevated even in case of marked cellular iron overload. Moreover, I also found the increase in the expression of TfR1 in treated cells. The concurrent over expression of TfR1, TfR2 and DMT1 –IRE could leads to their cooperation in

supporting iron cellular influx and this could justify the amount of iron in substantia nigra from PD affected patients, promoting iron toxicity (figure 24). This condition could be aggravated by the impairment of iron export. In fact, recent observations show that tau-knockout mice develop age-dependent brain atrophy, iron accumulation and substantia nigra neuronal loss, with concomitant cognitive deficits and parkinsonism. Amyloid precursor protein (APP) ferroxidase activity couples with surface ferroportin to export iron, but its activity is inhibited in Alzheimer's and Parkinson's diseases, thereby causing neuronal accumulation. In primary neuronal colture, it was found that a loss of tau also causes iron retention, by decreasaing surface trafficking of APP (Lei P *et al*., 2012).

The importance of the preliminary data reported here can be supported by the observation that, therapeutically targeting these pathways therefore may be a novel therapeutic avenue that obviates the need for systemic iron chelation that often results is very severe side effects. In fact, blocking one or both studied microRNAs, this could lead to a reduction in dopaminergic neurons iron uptake in PD affected patients. Moreover this study could be the first important answer to the question: "How TfR2 expression is regulated if it is surely not regulated by IRPs on the contrary of what happens for TfR1?".

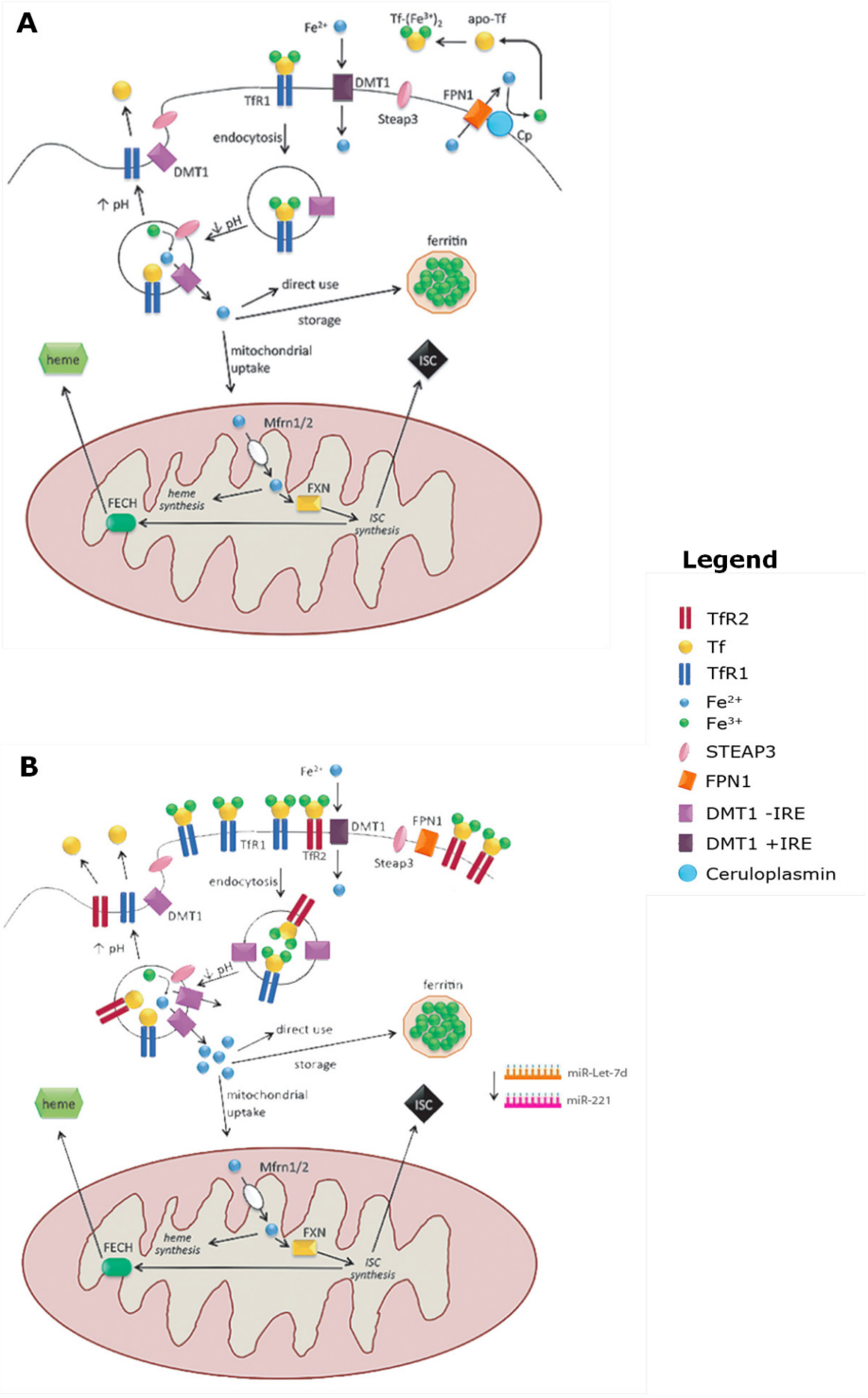

Figure 24. Model of iron overload in PD. Panel A shows what happens in normal condition while in a cellular model of PD (panel B) a low expression of microRNA-221 and microRNA-Let-7d was found. This phenomenon causes the up-regulation of both DMT1 –IRE and TfR2. An increase in the expression TfR1 was also found. All the three mentioned proteins are involved in iron uptake endosome mediated. Moreover, recent evidences show that in PD the iron export activity of FPN1 is reduced. All these factors contribute to the iron accumulation and to its subsequent toxicity.

Anyway further studies are necessary to confirm these data. Several other studies have been planned, such as the *in vitro* inhibition of the endogenous studied microRNAs and the validation on other PD *in vitro* models. Concerning to the use of animal models, an idea could be to create the commonly used *in vivo* model of PD treating mice with MPTP (the MPP^+ precursor) and to evaluate the microRNA-Let-7d and microRNA-221 expression after the treatment in their substantia nigra.

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