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## 'Study of novel pathogenic mechanisms in Huntington disease'

TESI DI DOTTORATO

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## **Introduction**

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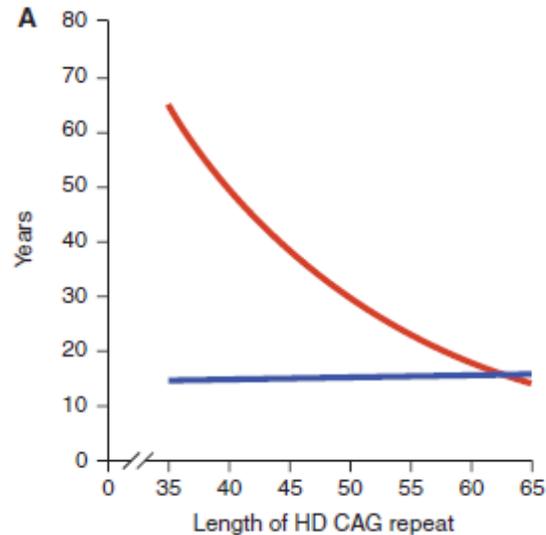
### **Huntington's Disease: a brief history**

In 1872 George Huntington first described the main clinical and inherited features of a neurological disorder, thus bearing his name (Huntington 1872), Huntington disease (HD). It is a devastating neurodegenerative and hereditary disorder associated with wide variability in neurological and psychiatric symptoms caused by an expanded CAG repeat in the gene coding for the huntingtin protein. About one century later, in 1983, a group of researcher in Boston identified a gene locus in linkage with chromosome 4p16.3 (Gusella et al., 1983) and, 10 years later, described the responsible gene with its mutation (Huntington's Disease Collaborative Research Group 1993) IT15, later called the HUNTINGTIN (HTT) gene. In the human genome there is not significant homology of IT15 to other genes; mRNA is approximately 10 kB size and encoded for a 350 kDa protein. A triplet nucleotide repeat, cytosine-adenonsine-guanine (CAG), is found near the 5' end of the gene's coding region and is translated into a polyglutamine (polyQ) stretch.

Since then, research efforts have exponentially increased our knowledge on the clinical, molecular and, recently, therapeutic features of the disease.

### **Huntington's Disease: a clinical overview of genotype-phenotype profile**

With the discovery of HTT, the clinical features of HD could be better defined by investigating genotype-phenotype relationships with greater specificity. Several studies focused on understanding the relationship between the age of onset (when an individual first develops neurological symptoms) and the length of the triplet repeat (Duyao et al. 1993; Rubinsztein et al. 1996; Snell et al. 1993) (Fig. 1). Alleles of HTT with fewer than 35 CAG repeats (CAGs) show no risk for causing HD. Individuals whit 36–40 CAGs may or not develop HD symptoms, becoming symptomatic later in the life.



**Figure 1.** The role of the CAG expansion in IT-15 on HD pathogenesis. Correlation of HD CAG-repeat length with age at onset. Best-fit curves for age at neurological onset (red) and duration of disease from onset to death (blue), plotted against CAG repeat length for the expanded mutant allele from Huntington disease (HD) patients. Age at onset is strongly correlated with the CAG-repeat length ( $r^2 \approx 0.54$ ;  $p < 0.001$ ), and duration of disease shows no correlation with the CAG-repeat length, suggesting that, after onset of HD, factors independent of the original trigger of pathogenesis determine the rate of progression. Adapted with permission from (Gusella and MacDonald 2006).

However, individuals with more than 40 CAGs repeats will probably develop symptoms if they live long enough. Curiously, individuals carrying two HD-causing alleles (i.e., homozygous for mutant HTT) appear to develop symptoms about the same age as people with a single allele and the same CAG expansion. However, homozygosity for the CAG mutation has been reported to lead to a more severe clinical course (Squitieri et al. 2003).

Interestingly, there is a striking and significant negative relationship between the length of the CAG expansion and the age of symptom onset; the longer the CAG stretch, the earlier symptoms typically appear (Fig. 1). The most common HD alleles contain 40–50 CAG repeats or polyQ. In that range, 50–70% of age of symptom onset appears to be explained by the length of the polyQ stretch, the remainder is determined by other modifying genes and environmental factors (Wexler et al. 2004). Length of the polyQ stretch also appears to influence the progression of pathology, although the link between disease progression and the length of the polyQ stretch appears to be weaker than for age of symptom onset (Brandt et al. 1996; Penney et al. 1997).

The correlation between the length of the CAG expansion and the clinical features of the disease represent the basis of the molecular mechanism of genetic anticipation (Telenius et al. 1993; Telenius et al. 1995). This anticipation theory in HD postulated that the male germ line inheritance of the mutant HTT allele often led to a more severe clinical course than the female germ line inheritance. On average, children develop HD symptoms about 8 years earlier than their fathers (Ranen et al. 1995). In those children, the CAG repeats had further expanded. The molecular mechanism responsible for this phenomenon is still under debate (MacDonald et al. 1993; Telenius et al. 1994; Telenius et al. 1995).

The length of polyQ stretch also affects HD symptoms. The most common HD alleles (40–50 CAGs) tend to produce classic disease symptoms that manifest during mid-life. HD symptoms include motor abnormalities, behavioural changes, progressive cognitive decline, weight loss, muscular sufferance and internal organ dysfunctions (Van der Burg et al., 2009). Although the clinical landmark of the disease is the symptom chorea, a non finalistic involuntary movement similar to an uncontrolled dance involving the whole body, many other symptoms characterise the disease course including dystonia, parkinsonism, gait disturbance, clumsiness, arm incoordination and ocular motor impairment (Penney et al., 1990). Although the most of cases show an age at onset around the 40s, the disease may rarely begin early in the life before 20 years of age (i.e. juvenile HD (JHD)) (Quarrell et al., 2009). In JHD CAG stretches is >50 and display symptoms very different from the adult onset. (Nance and Myers 2001). Unlike adult-onset HD, JHD is associated with a paucity of movements (bradykinesia) and an increased incidence of seizures.

Although the CAG number accounts for about 60-70% of age at onset variation, disease onset and progression depends also on other factors of biological or environmental origin or both (Squitieri et al., 2000, Wexler et al., 2004). Only when the mutation is highly expanded and toxic (i.e. beyond 60 CAG repeats), the disease sometimes begin in children even younger than 10 years of age and take a particularly devastating course (Quarrell et al., 2009).

The wide variability in symptoms depends on HD neuropathology extending from cortical brain areas (Rosas et al., 2008) to the striatum, the most and first affected brain structure (Aylward et al., 2004). Besides affecting the brain thus causing clinical manifestations, HD also damages peripheral tissues owing to the ubiquitously expressed htt protein (Van der Burg et al., 2009). Although the cause of these peripheral

symptoms remains unclear, growing evidence indicates that they arise because mutant htt acts directly on peripheral tissues (Van der Burg et al., 2009). A recent study reports a positive correlation between serum branched chain amino acid levels (valine, leucine and isoleucine), weight loss and disease progression in patients with HD, reinforcing evidence that HD induces major systemic metabolic defects (Markianos et al., 2005). Patients with HD also have numerous endocrine abnormalities, including abnormal corticosteroid (Leblhuber et al., 1995, Bjorkqvist et al., 2006) and reduced testosterone levels (Markianos et al., 2005). Peripheral deficits include also changes in growth factor levels (Battaglia et al., 2001, Ciammola et al., 2007) abnormal A(2A) receptor binding in blood cells (Varani et al., 2003; Maglione et al., 2005) changes in pro-catabolic serum profiling and plasma metabolites (Underwood et al., 2006; Forrest et al., 2010) immune protein up-regulation (Forrest et al., 2010; Bjorkqvist et al., 2008) and neuroendocrine dysregulations (Saleh et al., 2009). Muscles are affected early in HD (Ciammola et al., 2009), type 1 red fibre loss contributing to the weight loss (Chaturvedi et al., 2009). Rather than involving the brain alone HD is therefore a complex systemic disorder, thus requiring researchers to focus on potential disease markers also in the periphery.

### **Huntingtin protein**

The role of huntingtin protein is still unclear and under several debates. Htt is a soluble protein ubiquitously expressed, particularly present in the central nervous system (CNS) (Cattaneo et al 2005). Inside the cell, the cytoplasmic protein is associated with several organelles, microtubules and vesicular membranes, interfering with intracellular trafficking, exocytosis and endocytosis process. The protein plays a critical role in the embryonic development as well as in the antiapoptotic process. Htt is often associated with proteins involved in synaptic functions (Cattaneo et al., 2001).

Different studies demonstrated that, if overexpressed, the normal protein it is able to protect against apoptosis and excitotoxicity in various systems (cultured striatal cells, primary cultures from HD mouse models or in vivo) (Leavitt, et al., 2006; Zhang et al., 2003). The polyQ stretch is released following proteolysis and is more toxic when cleaved than when bound to the whole protein. This has been demonstrated in vivo in the first developed genetic model of HD, the R6/2 mouse, generated by the expression of 150 CAG repeats of exon 1 of the human mHtt gene (Mangiarini et al., 1996). The

released polyQ stretches trigger the formation of aggregates and alter the Htt structure, thereby leading to its cytotoxicity.

The cytotoxic effect of mHtt takes various forms that affect several cellular pathways and functions. The pleiotropic effects of mHtt cytotoxicity have been reported in a number of HD models (*Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*) and in various cell populations using gene arrays (Sugars et al., 2003; Sassone et al., 2009). The exact mechanisms involved have not yet been clarified, but it is now well known that post-translational modifications can alter the function of the protein and that the mutated protein (mHtt) interferes with the regulation of gene transcription.

The fact that HD is an autosomal dominant disorder argues in favor of a “gain of function” of the protein hypothesis, where mHtt is characterized by a deleterious functions relevant for HD pathogenesis. In this view, the mutated form of Htt acquires the ability to interact with several proteins (e.g., huntingtin-associated protein (HAP1), huntingtin-interacting protein (HIP1), G-protein-coupled receptor kinase-interacting protein (GIT1), postsynaptic density protein (PSD95) etc.(Cisbani and Cicchetti 2012). In vivo studies demonstrated that loss of a single Htt allele is not predictive of pathological phenotype. The presence of the normal allele in pathological conditions does not improve the phenotype. Squitieri et al. in 2003 demonstrated that homozygous and heterozygous HD patients are phenotypically indistinguishable (Squitieri et al., 2003).

However, the presence of mHtt can also lead to a “loss of function”: mitochondrial motility is altered, probably immobilized in neurons as seen in HD models (Trushina et al., 2004). Moreover the synthesis and the release of brain-derived neurotrophic factor (BDNF) is strongly impaired, significantly contributing to poor neuronal survival. The function of normal Htt can further be challenged if sequestered by the mutant protein. Considering all these functions and, in particular, its role in embryonic development, the normal protein is essential for proper function of the organisms as also showed in a conditional knockout model of the normal gene where neuronal degeneration in the postnatal brain is triggered (Dragatsis et al., 2000). On the other hand, the absence of the expanded CAG repeats caused high levels of autophagy in vitro and a consequent mHtt aggregates remission (Zheng S, et al., 2010). Thus, the presence of the expanded polyQ stretch may inhibit the normal function of Htt in the autophagy process (Cisbani and Cicchetti 2012).

We can summarize that there are evidences confirming both of theories: a gain of toxic function and a loss of normal function of Htt. In HD pathogenesis, the loss of normal Htt function and the altered functions caused by mutated protein may act in concert and contribute to the course of the disease. We can hypothesize a cross-talk between the two Htt forms, which would result in a negative-dominant effect of the altered protein (Zuccato et al., 2001). Thus, the theory that Htt may undergo both a loss and a gain of function is progressively becoming a prevalent view in the literature (Cattaneo et al., 2001).

### **Neuropathology and excitotoxic mechanisms**

The pathological hallmark of HD is the progressive atrophy of the striatum (caudate nucleus, pallidus and putamen), due to the spiny gabaergic neuron death. Indeed the most commonly used grading system to assess the severity of HD degeneration (developed by Vonsattel et al., 1985) is based on the pattern of striatal degeneration in post-mortem tissue; it classifies HD cases into five different severity grades (0–4), with a Grade 0 indistinguishable from normal brains. Interestingly, the degree of striatal atrophy also correlates with the degeneration of other non-striatal brain structures: intracellular dysfunction induced by mutant huntingtin progressively leads to the degeneration of important neuronal pathways and cell loss in the striatum, but also in cerebral cortex and other brain regions (Vonsattel & DiFiglia 1998; Kremer et al., 1990, 1991; Sapp et al., 2001; Kassubek et al., 2004; Petersén et al., 2005).

Various mechanisms such as excitotoxicity, metabolic impairment, mitochondrial dysfunction, oxidative stress, apoptosis, abnormal production and transport of neurotrophic factors and autophagy have been implicated in HD pathology (Beal et al., 1986; Ross 2004; van der Burg et al., 2009). Many of these mechanisms, including astrogliosis, may slowly develop and become increasingly pronounced in the late stages of the disease. In particular, microglia activation reflects a progressive process of neuroinflammation, started during the asymptomatic stage of HD and increased over-time with the severity of the pathology (Sapp et al., 2001; Pavese et al., 2006; Tay et al., 2007).

Interestingly, activated astrocytes and microglia can critically regulate processes of neuronal death and survival by secreting glutamate, neurotrophic factors, and pro- and anti-inflammatory cytokines (Battaglia et al., 2011; Shin et al., 2005). The capacity of

the glial cells to remove extracellular glutamate from the synaptic cleft can also contribute to the vulnerability of striatal neurons to excitotoxicity. It was reported that mutant Htt accumulates in the nucleus of glial cells in HD brains, decreasing the expression of the glutamate transporter. Moreover, while wild-type glial cells protect neurons against mutant Htt-mediated neurotoxicity, glial cells-expressing mHtt do increase mutant Htt-induced neuronal vulnerability, suggesting that a decrease in glutamate uptake by glial cells may indeed exacerbate neuronal excitotoxicity in HD (Shin et al., 2005). Such decrease in glutamate uptake can be detected before any other evidence of neurodegeneration, suggesting that a defect in astrocytic glutamate uptake is an early event in HD (Liévens et al., 2001).

### **Mutant htt and neurotrophic factor: the role of TGF- $\beta$ 1**

Mutant Htt loses the ability to increase brain-derived neurotrophic factor (BDNF) gene transcription (Zuccato et al., 2001, 2003) and fails to enhance intracellular trafficking of BDNF (Gauthier et al., 2004; Del Toro et al., 2006). Strategies to increase brain levels of BDNF or of glial-derived neurotrophic factor (GDNF) result to be neuroprotective in HD models (Bemelmans et al., 1999; Perez-Navarro et al., 1999; Duan et al., 2003; Kells et al., 2004; McBride et al., 2003; 2006; Pineda et al., 2007; DeMarch et al., 2008; Gharami et al., 2008; Squitieri et al., 2008a). An unbalance between neurotoxic and neuroprotective factors may result in neuronal dysfunction and cell death in HD. Emerging evidence suggests that the study of neurotrophic factors in HD can be extended to the periphery in an attempt to obtain accessible indicators of disease progression and drug efficacy.

Among all the neurotrophic factors, TGF- $\beta$ 1 plays an important role in the regulation of numerous processes including cell cycle control, regulation of early development, cell differentiation, extracellular matrix formation, hematopoiesis, angiogenesis and immune functions (Dennler et al., 2002). In addition to its role in the regulation of neuronal survival, TGF- $\beta$ 1 interferes with several molecular mechanisms potentially related also to HD such as: expression of proteases (specifically caspase 3 and 11) (Kim et al., 2004), protein fragment accumulation (specifically PrP and amyloid deposition) (Boche et al., 2006; Tesseur and Wyss-Coray 2006), microglial cells reactivity (Kim et al., 2004; Boche et al., 2006), glucose uptake (Kitagawa et al., 1991) and regulation of

protective neurotrophines such as BDNF and GDNF, which have been previously proved to be altered in HD (Zuccato et al., 2001; McBride et al., 2006). Both BDNF and GDNF require TGF- $\beta$ 1 to exert neuroprotective effects (Unsicker and Kriegstein, 2000, 2002; Schober et al., 1999). Lack of TGF $\beta$ 1 strongly reduces survival in primary neurons cultured from TGF- $\beta$ 1 -null mice and increases the susceptibility to excitotoxic injury. Consistently, over-expression of TGF- $\beta$ 1 in astroglial cells protects adult mice against neurodegeneration in acute, excitotoxic and chronic injury paradigms (Bruno et al., 1998; Brionne et al., 2003).

TGF- $\beta$ 1 is predominantly synthesized by neurons and glial cells, within the Central Nervous System (CNS), and by platelets and monocytes/macrophages in the peripheral tissues (Prud'homme, 2007; Mosser et al., 2008). Macrophages display remarkable plasticity that enables them to perform distinct and even opposing function, such as release of either inflammatory or anti-inflammatory cytokines and growth factors, in response to different environmental cues (Stout et al., 2005). Depending on the activation state, macrophages can be designed as either classical activated (M1), with proinflammatory properties, or alternatively activated (M2) cells, which mediate anti-inflammatory response (Gordon, 2003). Under physiological condition, macrophages, like glia, interact with their surroundings and provide protective cytokines and neurotrophins. Upon insult, both cell populations can become pathologically activated leading to neuro-inflammation, and/or neurodegeneration by altering expression of many neurotrophic factors (Lucin et al., 2009; Lynch, 2009).

However, the presence of abnormalities is not restricted to the CNS, but found in several other compartments including the neuroendocrine system, skeletal muscles, cardiovascular system, blood, digestive tract, thyroid, skeleton and genital organs (Van der Burg 2009)

At the cellular level, the loss of function of the wild-type protein and toxic gain of function of the mHtt both contribute to the disruption of multiple intracellular pathways, including postsynaptic signaling, apoptosis, transcriptional regulation, protein trafficking and vesicular transport. Consequently, mHtt induces a cascade of events involving excitotoxicity, proteasome impairment, mitochondrial dysfunction, oxidative stress, metabolic impairment, calcium homeostasis dysregulation, apoptosis and autophagy (Gil JM, 2008). The combination of these intracellular dysfunctions over time causes the progressive neuronal degeneration that characterizes HD (Borrell-Pages 2006).

Understanding this systemic nature of HD is important for better developing models of disease pathogenesis to design disease modifying therapies, as currently the condition can only be treated with drugs that offer varying degrees of symptomatic benefits (Wright BL, 2007; Phillips W, 2008; Frank S, 2010)

## **Rationale**

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Astrocytes and microglia as well as neurons are the major producers of TGF- $\beta$ 1 in injured brain (Finch et al., 1993; Flanders et al., 1998). TGF- $\beta$ 1 contributes to maintain neuronal survival and integrity of CNS and to regulate microglial activation (Brionne et al., 2003). Perturbations of the TGF- $\beta$ 1 signaling are involved in many neurodegenerative disorders. An aberrant expression of TGF- $\beta$ 1 receptor II (TGFRII) has been reported in the brain of Alzheimer's disease (AD) patients (Tesseur et al., 2006; Ueberham et al., 2006). Reduced TGF- $\beta$ 1 signaling increases amyloid deposition and neurodegeneration in transgenic AD mice (Tesseur and Wyss-Coray 2006). The role of TGF- $\beta$ 1 has been also investigated in several other neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) (Ilzecka et al. 2002), Parkinson disease (PD) and Prion diseases (Boche et al., 2006). Reduced levels of TGF- $\beta$ 1 in the brain increase susceptibility to excitotoxic injury and neurodegeneration in heterozygous TGF- $\beta$ 1 knockout mice (Brionne et al., 2003). TGF- $\beta$ 1 production is under the control of extracellular signals including estrogens and excitatory amino acids acting at mGlu3 metabotropic glutamate receptors (Bruno et al., 1998; Sortino et al., 2004). Interestingly, the activation of glial mGlu3 receptors is reported to be neuroprotective via a paracrine mechanism mediated by TGF- $\beta$ 1 (Bruno et al., 1998; D'Onofrio et al., 2001; Corti et al., 2007). mGlu3 receptors, expressed by astrocytes, are able to control the production of TGF- $\beta$ 1, suggesting that the activation of glial mGlu3 receptors promotes a form of glial-neuronal interaction mediated by the synthesis and release of such neuroprotective factors (Bruno et al., 1998; D'Onofrio et al., 2001; Corti et al., 2007). Interestingly, the expression of mGlu3 receptors is substantially reduced in the striatum of R6/2 mice (Cha et al., 1998), and TGF- $\beta$ 1 signaling is reduced in vitro in cells expressing HD mutation (van Roon-Mom et al., 2008).

Importantly, we have recently shown that TGF- $\beta$ 1 plays a critical role in the pathogenesis of HD during the presymptomatic disease stage (Battaglia et al., 2011). Our data showed an early defect of TGF- $\beta$ 1 in HD brain and peripheral tissues. Such defect was remarkable at the beginning of the disease in the several *in vitro* and *ex vivo* models we analyzed (Battaglia et al., 2011). Specifically, TGF- $\beta$ 1 was defective in the cerebral cortex of human autaptic brain samples from HD subjects and of two different transgenic mouse models (R6/2 and YAC128). Such defect was confirmed also at mRNA and protein level in murine astroglial cells transfected with exon-1-Htt-72Q-EGFP, in knock-in neuronal cells (Trettel et al., 2000) expressing wild-type (STHdh7/7Q) and mutant Htt (STHdh111/111Q) before and after transfection with exon-1-Htt-72Q-EGFP (Battaglia et al., 2011) as well as in immortalized glial cells (SVG-p12) after transfection with exon-1-Htt-72Q-EGFP. Coherently with previous works (Cha et al., 1998), we confirmed a reduced mGluR2/3 expression in transgenic brain mice and, as a direct consequence of it, we found a defective TGF- $\beta$ 1 release due to impaired mGluR expression, after stimulation with the receptor agonist LY379268, which failed to increase the cytokine level in HD compared to control mice. Our first data correlated the TGF- $\beta$ 1 deficiency with clinical genetic parameters (i.e. cytokine levels in serum correlating with HD stages, expanded CAG repeat number, disability scale score, human brain glucose uptake) and indicated a relative increase of the cytokine during HD development, coherently to the increasing inflammatory process as we have observed in autaptic human brain with HD mutation. All together, these data encourage TGF- $\beta$ 1 alteration as a possible new mechanism as for AD and other neurodegenerative diseases, potentially related to neuroprotection and neuroinflammation.

## Aim

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The aim of this study was to elucidate whether the aberrant production of TGF- $\beta$ 1 might represent an inflammation-related process in both central and peripheral district useful for monitoring disease development and potentially suitable for predicting disease onset in HD.

project end points were to investigate:

1. whether changes of TGF- $\beta$ 1 levels in HD depend on the variation in the percentage of TGF- $\beta$ 1 producing-monocytes/macrophages along HD course. These experiments were conducted in HD human cells and relative controls. We get this first end point analysing the content of TGF- $\beta$ 1 in monocytes from HD patients whole blood.
1. whether the differential capacity of macrophages to produce TGF- $\beta$ 1 reflects distinct cell phenotypes during disease stages. We get this second end point analysing the expression of specific surface receptor expressing in 2 different macrophages sub-population (M1 and M2) by flow cytometry technique.
2. whether changes of nuclear factor-kB (NF-kB)-p65 expression may likely represent a molecular mechanism governing macrophages heterogeneity in HD. We get this third end point analysing the expression of this protein in isolated monocytes from different stage HD patients by immunoblot.
3. whether such abnormal production of the cytokine in the periphery mirrors what we may observe in the central nervous system. This experiment will be conducted by immunohistochemical analysis on human autaptic brain samples obtained from HD subjects and healthy controls from Columbia University Brain Bank, New York, USA.
4. what cell population is involved in the abnormal production of TGF- $\beta$ 1 in the brain (i.e. glial and/or neuronal cells). For this purpose, immunohistochemical analyses will be conducted.
5. whether there is any correlation between central and peripheral abnormal TGF- $\beta$ 1 levels. We will relate the abnormal TGF- $\beta$ 1 levels to the indices of HD development such as UHDRS scores, HD burden scores and a new

developmental index taking into account years to/from age at onset in at risk people (Di paola et a., 2012, online; Sanchez-Castagneda et al, 2012, in press).

## Methods

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### *Subjects*

A total of 112 HD subjects (14 preHD, 15 stage I, 36 II, 47 III-V), and 46 gender- and age-matched healthy controls divided into 3 groups: 25-40, 41-55, and 55-80 year-old were recruited. Subjects' demographic, clinical and genetic characteristics are reported in Table 1. PreHD subjects had previously requested a pre-symptomatic genetic test by entering a specific program whose protocol was ethically approved (Squitieri et al., 2008b). All HD subjects revealed a CAG repeat expansion mutation and all of them as well as controls were required to sign an informed consent before recruitment in the study. All human experiments were performed in accordance with the Declaration of Helsinki (Declaration of Helsinki, 1991).

Control subjects with a suspect of cardiovascular, psychiatric or neurodegenerative disorders other than HD, were excluded from this study. Clinical examinations were conducted using the Unified Huntington's Disease Rating Scale (UHDRS) to measure motor, cognitive, behavioral and general function (Huntington Study Group, 1996) and the Mini-Mental State Examination (MMSE) was used to screen general cognitive function (Huntington Study Group, 1996; Folstein et al., 1975).

PreHD subjects included either asymptomatic individuals (total motor score of  $< 5$  in the UHDRS and cognitive and behavioural assessment within the normality) or individuals with soft signs (suspicious clinical features that were insufficient to warrant a diagnosis of HD) (Squitieri et al., 2003). The patients' age at onset (AO) was retrospectively established by interviews to family members regarding the first neurological manifestations (Paulsen et al., 2001), thus excluding, in this first study stage, subjects showing severe cognitive and psychiatric abnormalities that represented a permanent change from the normal state (Brinkman et al., 1997). The predicted years to manifest the disease were calculated on the basis of the survival analysis formula described by Langbehn et al. (2004) (Langbehn et al., 2004). To estimate the progression of the pathological process from pre-HD stage, we calculated the 'HD development' index by combining the predicted years to onset for preHD subjects and disease duration (years-from-onset) for patients (Di Paola et al., 2012, Sánchez-Castañeda et al., 2012). The disease burden (DB) index was measured according

to the previously described formula:  $\text{age} \times (\text{CAG} - 35.5)$  (Penney et al., 1997). The Disability Scale (DS) combines patients' independence and motor performance, thus taking into account the subjects' independence on neurological motor impairment (Myers et al., 1991). The disease stage was calculated according to the Total Functional Capacity (TFC) score (Marder et al., 2000).

**Table 1:** Demographic and clinical data of healthy controls and HD patients

Subjects	Number (M,F)	Age (24-77yrs)	TFC (13-0)	CAG repeats (39-55)	AO (16-69)	HD DI(yrs) (52,53)	DB (108-759)	DS (100-0)
Pre-HD	14 (M=8, F=6)	35,4 ± 4,4	13	44,0 ± 2,4	-	-13,8 ± 7,9	296,9 ± 70,8	99,7 ± 0,9
I HD	15 (M=9, F=6)	46,9 ± 8,4	11,8 ± 1,1	44,1 ± 2,2	44,2 ± 8,1	-1,6 ± 6,2	389,5 ± 70,2	92,1 ± 4,1
II HD	36 (M=17, F=19)	55,4 ± 10,9	7,7 ± 0,8	43,2 ± 3,0	47,1 ± 10,3	3,9 ± 10,3	403,8 ± 124,1	77,2 ± 6,3
III HD	31 (M=18, F=13)	53,6 ± 11,7	4,6 ± 1,3	44,5 ± 3,5	44,1 ± 10,1	6,7 ± 6,7	448,8 ± 110,1	61,3 ± 11,6
IV HD	10 (M=4, F=6)	59,2 ± 10,5	1,6 ± 0,5	44,0 ± 3,6	46,4 ± 10,2	11,7 ± 6,1	470,7 ± 128,0	39,0 ± 8,4
V HD	6 (M=2, F=4)	58,3 ± 13,4	0	45,0 ± 5,3	41,0 ± 15,6	10,3 ± 5,9	499,5 ± 127,0	22,9 ± 6,4
	<b>UHDRS1 (1-100)</b>	<b>UHDR S2 (248-7)</b>	<b>UHDRS 3 (0-88)</b>	<b>UHDRS4 (0-24)</b>	<b>UHDRS 4 (0-24)</b>			
Pre-HD	5,8 ± 4,3	278,3 ± 33,1	6,2 ± 6,9	25 ± 0	25 ± 0			
I HD	24,1 ± 7,9	186,6 ± 54,1	16,5 ± 7,5	23,7 ± 1,7	23,7 ± 1,7			
II HD	39,3 ± 12,2	132,2 ± 52	19,7 ± 7,9	17,6 ± 4,2	17,6 ± 4,2			
III HD	55,4 ± 12,8	89,1 ± 48,8	23,4 ± 10	9,8 ± 5,1	9,8 ± 5,1			
IV HD	75,8 ± 21,4	71,3 ± 44,9	23,3 ± 14,2	1,3 ± 0,8	1,3 ± 0,8			
V HD	90,2 ± 13,4	-	15 ± 6	0,2 ± 0,4	0,2 ± 0,4			

Control subjects are 46 (M=19,F=27, mean age 49,3 ± 10,7). Values are given as mean ± s.d. yrs: years; TFC: total functional capacity; AO: age at onset; HD DI: Huntington Disease Development Index; DB: Disease Burden; DS: Disability Score; UHDRS: Unified Huntington Disease Rating Scale; MMSE: Mini Mental State Examination

### ***Human macrophages***

Isolation and differentiation. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Ficoll–Hypaque) from freshly drawn venous blood (platelet-free). The cell pellet was layered on a Percoll (GE Healthcare) gradient and the interphase containing monocytic cells was obtained following a 800g centrifugation (Gantner et al., 1997). After washing monocytes were seeded into 24-

well plates culture containing RPMI (Sigma, San Louis, MO, USA) supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine and 100 µg/ml streptomycin and 100 units/ml penicillin. Cells were maintained for 7 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and cultured in presence of 100 ng/ml of macrophages colony stimulating factor (M-CSF, GE-Healthcare) to obtain fully differentiated macrophages (Darwich et al., 2008).

Flow cytometry analysis. Adherent macrophagic cells were detached by gently pipetting cold phosphate buffered saline (PBS), centrifuged at 200× g for 5 minutes and washed with PBS. Cell suspension was then fixed with 4% PFA and incubated with FITC-conjugated anti-human CD80 (EuroBioSciences GmbH, Germany) or an equivalent amount of isotypic control IgG1 (EuroBioSciences GmbH, Germany) for 1 h. CD80-positive macrophages were >90% gated cells (data not shown). For the detection of intracellular cytokines, cells were permeabilized with a buffer containing 0.1% (w/v) tritonX-100, 0.05% (w/v) NaN<sub>3</sub> in PBS, and incubated for 1 h at room temperature with PE-conjugated anti-human TGF-β1 or PE-conjugated anti-human IL-10 or antihuman IL-12 (BD Pharmingen, San Jose, CA USA) or an equivalent amount of IgG1 and IgG2a (BD Pharmingen, San Jose, CA USA) isotypic controls, respectively. Cells were washed and resuspended with PBS before acquisition. The cytokine-positive cells and the relative MFU were scored on the basis of isotype controls.

### ***Real-time RT-PCR analysis***

TGF-β1 gene expression in macrophages from preHD subjects, HD patients and healthy controls, was measured by quantitative PCR analysis performed on a StepOnePlus instrument (Applied Biosystems) by using the following primer sequences: Fwd: 5'-CGAGCCTGAGGCCGACTACTA-3'; Rev: 5'-CTCGGAGCTCTGATGTGTTGAA-3'. Briefly, total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's instructions and reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo-d(T) primer. Resulting cDNAs were amplified using Power SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturers' instructions. The level of each mRNA was normalized to that of cyclophilin A (CypA). PCR cycling parameters were as follows: 50°C for 2 min, 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 60°C for 1 min, and 72°C for 40 s.

### ***Cell subsets from whole blood***

Whole blood analyses were carried out in 81 HD individuals (5 preHD subjects, 11 I HD, 36 II HD and 29 III-V HD stage patients) and 26 gender- and age-matched healthy controls. Venous blood drawings were collected applying minimal venous stasis in order to minimize cell activation. Whole blood (50  $\mu$ l) for each sample was fixed with PFA 2% over night at 4°C and circulating cell subsets were then gated by using forward and side light-scatter patterns (Fig. 2A). Cells were then labelled with FITC-conjugated anti-human CD14 (BD Pharmingen, San Josè, CA USA) or equivalent amount of isotypic control IgG2a,k (BD Pharmingen, San Josè, CA USA) for the detection of monocytes. CD14-positive monocytes were >90% gated cells (data not shown). After washing with phosphate buffered saline (PBS), cells were permeabilized with 0.3 % tritonX-100 and incubated with PE-conjugated anti-human TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN USA) or an equivalent amount of isotypic control IgG1 (R&D Systems, Minneapolis, MN USA). The percentage of TGF- $\beta$ 1<sup>+</sup> cells and the intracellular content of the cytokine, reported as Mean Fluorescent Unit (MFU) were scored on the basis of isotype control. All the experiments were performed by using a Becton-Dickinson FACS Calibur flow cytometer (BD, San Josè, CA, USA).

### ***Flow cytometry analysis of macrophage M1 and M2 subsets***

Analyses of macrophage subsets were carried out in 12 HD individuals (5 early HD subjects and 7 II-III HD stage patients) and 5 gender and age-matched healthy controls. After isolation and differentiation, adherent macrophagic cells were detached using trypsin-EDTA and centrifuged in RPMI at 200 $\times$  g for 10 minutes and washed with PBS. Cell suspension was then incubated with FITC-conjugated anti-human CCR2 and with PE-conjugated anti-human CX3CR1 or equivalent amount of isotypic control IgG2b and IgG1, respectively. Cells were incubated for 45 minutes at 4°C in the dark, washed with PBS and acquired to the cytometer. Macrophages were gated on the basis of cells size (FSC-H) and cells density (SSC-H). The percentage of M1 macrophages was identified as CCR2 positive-CX3CR1 negative cells (CCR2<sup>+</sup>CX3CR1<sup>-</sup>) while the percentage of M2 macrophages was identified as CCR2 negative -CX3CR1 positive cells (CCR2<sup>-</sup> CX3CR1<sup>+</sup>) on the basis of isotype controls. All antibodies were purchased from R&D Systems (Minneapolis, MN USA). All the experiments were performed by using a Becton-Dickinson FACSCalibur flow cytometer (BD, San Josè, CA, USA).

### ***NF- $\kappa$ B-p65 immunoblotting***

Monocytes-derived macrophages from preHD subjects, symptomatic HD patients and controls were lysed in lysis buffer containing 20 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 20 mM NaF, 2 mM Na<sub>3</sub>V0<sub>4</sub>, and 1:1000 protease inhibitor mixture (Sigma-Aldrich), sonicated with 2 × 10 s pulses and then centrifuged for 10 min at 10,000 × g. For analysis of NF- $\kappa$ B-p65 protein levels, total protein lysate was immunoblotted with anti-NF- $\kappa$ B-p65 (C-20) (1:500) (Santa Cruz, sc-372).

HRP-conjugated polyclonal secondary antibody (GE-Healthcare) was used at 1:5000 dilution. Protein bands were detected by ECL Prime (GE Healthcare) and quantitated with Quantity One (Bio-Rad Laboratories) and/or ImageJ software.

### ***TGF- $\beta$ 1 in human post-mortem brain samples***

We examined post-mortem brain tissues from 10 patients at different pathological grades of HD (Vonsattel et al., 1985) and three healthy controls. Samples were obtained by the New York Brain Bank at Columbia University, New York, USA. Clinical and neuropathological data were summarized in Table 2. Formalin fixed, paraffin-embedded striatal tissues were sectioned at 10  $\mu$ m. Deparaffinized sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were treated with Pronase at 37°C for 10 min for antigen retrieval and incubated overnight with monoclonal mouse anti-TGF-  $\beta$ 1 antibody (1:1000; Chemicon, CA). TGF-  $\beta$ 1 expression was detected by incubating the sample for 1 hour with secondary biotinylated antimouse antibody (1:200; Vector Laboratories, Burlingame, CA). Visualization of the immunoreaction was performed with 0.05% 3,3'-diaminobenzidine tetrachloride (DAB) (ABC Elite kit; Vector Laboratories). Control staining was performed without the specific primary antibody.

Double fluorescence immunohistochemistry was performed by incubating brain sections overnight with polyclonal rabbit anti-TGF- $\beta$ 1 antibody (1:100; Sigma-Aldrich) and monoclonal mouse anti-Glial Fibrillary Acid Protein (GFAP) (1:300; Sigma-Aldrich). Proteins were then visualized after 1 hour of incubation with secondary Cy3 anti-rabbit (1:200 Chemicon) and fluorescein anti-mouse (1:100; Vector Laboratories) antibodies.

**Table 2:** Pathological and clinical data of three control subjects and ten HD patients analysed for TGF-B1 expression in the striatum post-mortem sample.

Patients		Age (years)	Gender	Vonsattel et al's grade (59)
1	Healthy control	74	M	CTRL
2	Healthy control	67	M	CTRL
3	Healthy control	79	F	CTRL
4	Symptomatic	69	M	I
5	Symptomatic	50	M	II
6	Symptomatic	58	M	II
7	Symptomatic	69	M	II
8	Symptomatic	53	M	III
9	Symptomatic	56	M	III
10	Symptomatic	53	M	III
11	Symptomatic	47	F	IV
12	Symptomatic	55	-	IV
13	Symptomatic	49	F	IV

CTRL= Healthy controls; I= early HD; II= light HD symptoms; III= moderate HD symptoms; IV= strong HD symptoms.

### *Statistical analysis*

Statistical differences in the percentage of TGF- $\beta$ 1 producing macrophages, among patients at different HD stages versus controls, were performed by Mann Whitney non-parametric test. Linear dependence of TGF-  $\beta$ 1<sup>+</sup> macrophages on Age at Onset (AO), Disease Burden (DB), Disability Scale (DS), Time from/to Onset (TO), UHDRS1, 2, 3, 4 scores and MMSE was determined by a simple regression model. Other statistical analyses were performed by Student's t-test. Data were considered statistically significant at  $p \leq 0,05$ . Statistical analysis was performed with Biostat2009 software.

## Results

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### *Abnormal levels of peripheral TGF- $\beta$ 1 in HD depend on monocytic/macrophagic cell subset*

We examined the contribution of each whole blood cell subset (lymphocytes, monocytes and granulocytes) to the TGF- $\beta$ 1 production. Flow cytometry analysis (FACS) showed similar absolute counts (n°cells x10<sup>3</sup>/ml) of lymphocytes, granulocytes and monocytes (subset) in HD individuals (Table 1) and controls (Fig. 2B-D).

Each cell subset was then examined to determine the relative contribution to TGF- $\beta$ 1 production. The percentage of lymphocyte and granulocyte-producing TGF- $\beta$ 1 was similar in HD subjects and healthy controls (Fig. 3A,B). Conversely, the percentage of TGF- $\beta$ 1-producing (TGF- $\beta$ 1<sup>+</sup>) monocytes was dramatically lower in pre-manifested (preHD) and clinical stage I subjects when compared with controls and advanced stage HD patients (Fig. 4A).

Analysis of TGF- $\beta$ 1 intracellular content, reported as mean fluorescence unit (MFU), indicated that cytokine expression did not differ significantly among all the groups (Fig. 4B). Similar to monocytes, macrophages from preHD and clinical stage I subjects showed only a small portion of TGF- $\beta$ 1<sup>+</sup> cells when compared to cells derived from advanced HD stage patients and controls (Fig. 5A,B). No difference in the number of TGF- $\beta$ 1<sup>+</sup> cells was detected either between severe symptomatic patients and normal control subjects (Fig. 5B), or between controls themselves (data not shown). Intriguingly, additional FACS analysis revealed that MFU values were significantly higher in macrophages from preHD individuals as compared to controls (Fig. 5C), indicating an increased expression of intracellular TGF- $\beta$ 1 per cell at the early stage of the disease. Consistently, analysis of gene expression showed a robust increase of TGF- $\beta$ 1 mRNA levels in preHD if compared either to control subjects or more advanced HD patients (Fig. 5D). No difference in both MFU and TGF- $\beta$ 1 gene expression was detected between later stage HD patients and controls (Fig. 5C,D).

### *Macrophages display distinct phenotypes throughout the disease course*

In an attempt to investigate whether dynamic change of TGF- $\beta$ 1 production during HD course was due to a phenotypic heterogeneity of macrophagic cells, we explored cell surface markers associated with either M1 or M2 phenotypes in macrophages from HD individuals and healthy controls. Interestingly, flow cytometry analyses revealed

diversity of macrophage subsets along disease course. In particular, preHD subjects and clinical I stage HD patients showed a preferential pro-inflammatory M1 phenotype, - high percentage of CCR2<sup>+</sup>CX3CR1<sup>-</sup> cells (Fig. 6A); conversely, macrophages from HD patients in the late stage of the disease, displayed changes in the expression of surface markers in favour of anti-inflammatory M2 phenotype - high frequency of CCR2<sup>-</sup>CX3CR1<sup>+</sup> cells - (Fig. 6B). Expression of M1 and M2 surface markers throughout the disease course was further confirmed by RT-PCR (data not shown).

***Diversity in macrophages phenotype is associated with differential production of interleukin (IL)-10 and IL-12 during HD course***

Beside cell surface markers, macrophages phenotype can be identified based on the production of specific cytokines (Mantovani et al., 2004). In this regard, we aimed to further confirm the diversity in macrophages phenotype in HD by examining the variation of specific cytokines production pattern at different stages of the disease. Consistent with macrophages heterogeneity during HD course, we found that the percentage of pro-inflammatory IL-12- producing (IL-12<sup>+</sup>) cells (M1) was significantly increased in the early stage of the disease compared to controls and it returned to control values in the late stages of the disease (Fig. 7A). The reduction in the frequency of IL-12<sup>+</sup> cells in symptomatic HD patients, was associated with a concomitant increase in the percentage of anti-inflammatory IL-10-producing (IL-10<sup>+</sup>) macrophages (M2 phenotype) (Fig. 7B). Despite such phenotypic diversity, however, the intracellular content of both IL-12 and IL-10 did not differ significantly among all the groups (data not shown).

***Changes of NF-κB-p65 expression contributes to macrophages heterogeneity throughout HD course***

In order to clarify the possible molecular mechanism underlying the differential pattern of macrophages activation along disease course, we investigated the potential involvement of NF-κB in the promotion of distinct macrophages phenotypes. Analysis of protein expression indicated that monocytes-derived macrophages from preHD subjects displayed higher levels of NF-κB-p65 compared to symptomatic HD patients, who instead showed protein levels similar to controls (Fig.8).

***TGF- $\beta$ 1 expression in human post-mortem striatum is disease stage-dependent***

Immunohistochemical analysis performed in post-mortem human brain striatum, obtained from HD subjects and healthy controls, showed that TGF- $\beta$ 1 immunoreactivity gradually increased with disease severity (Fig. 9, Table 2). TGF- $\beta$ 1 immunoreactivity was first detected from pathological grade II HD brain tissues and reached a peak in grade IV HD brains (Fig. 9). Furthermore, fluorescent immunohistochemical analysis showed progressive reactive gliosis, as indicated by gradual increase of GFAP immunoreactivity in HD brain tissues along disease course. Dual immunofluorescence showed co-localization between TGF- $\beta$ 1 and GFAP positive cells starting from grade II HD patients up to later stages (Fig. 10). GFAP positive cells did not show any TGF- $\beta$ 1 immunoreactivity in controls and pathological grade I HD tissues (Fig. 9).

***Percentage of TGF- $\beta$ 1 + macrophages correlates with clinical and genetic parameters***

When we explored possible relationships between TGF- $\beta$ 1 and clinical parameters, we observed a statistically significant positive correlation between the percentage of TGF- $\beta$  + cells and age at onset (Fig. 11A), disease burden (Fig. 11B), HD development index (Fig. 11C), as well as the motor performance score (UHDRS1) (Fig. 11D). A significant negative correlation was also found with disability scale (Fig. 11E), functional test scores (UHDRS4) (Fig. 11F) and cognitive test scores (UHDRS2 and MMSE) (Fig. 11G,H). Instead, we did not find any correlation between TGF- $\beta$ 1+ cells and the behavioural test score (UHDRS3) (Fig.11I).

## Discussion

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Changes in the levels and the activities of endogenous neurotrophic factors are considered critical for the progression of degeneration in CNS diseases (Connor and Dragunow, 1998; Kruttgen et al., 2003; Murer et al., 2001), including HD (Zuccato et al., 2005). Defective bioavailability of such factors may have profound impact on the homeostasis of the brain, affecting neuroplasticity and leading to neuronal death (Zuccato et al., 2001). Recently, we have reported dynamic variations of TGF- $\beta$ 1 levels throughout the natural course of the disease and argued that the defective bioavailability of the cytokine early in the disease might contribute to the development of HD (Battaglia et al., 2011).

In the present study, we show that changes of TGF- $\beta$ 1 levels in the periphery may be attributable to an aberrant production by peripheral monocytes/macrophages. The number of TGF- $\beta$ 1-producing cells varied with disease progression and displayed a profile that was similar to the pattern of TGF- $\beta$ 1 levels in the serum of HD patients (Battaglia et al., 2011). Therefore, lower number of TGF- $\beta$ 1<sup>+</sup> monocytes/macrophages in preHD subjects, might potentially explain the reduced bioavailability of TGF- $\beta$ 1 in the serum of HD individuals at similar clinical stage.

Both peripheral monocytic and macrophagic cell subsets from preHD subjects showed a significant reduction of TGF- $\beta$ 1<sup>+</sup> cells when compared to controls and late stage HD patients. The lower percentage of TGF- $\beta$ 1<sup>+</sup> macrophages was associated with an increased intracellular content of the cytokine in preHD subjects and was accompanied by remarkable increase in TGF- $\beta$ 1 gene expression, suggesting an attempt of cells to counteract the early defective production of the trophic factor. Macrophages represent a heterogeneous cell population that exhibits remarkably plasticity and can change functional state in response to microenvironmental cues (Cassetta et al., 2011).

Based on their activation state, macrophages can be divided into two polarized phenotypes known as M1, or “classical activated” and M2 or “alternative activated” macrophages (Gordon, 2003; Mantovani et al., 2005). Identification of distinct macrophages subsets with divergent effects is based on the expression of transcription factors, cell surface markers that provide a mechanism for their differential recruitment in response of different signal and production of specific cytokines (Mantovani et al., 2004; Sica and Mantovani, 2012). M1 macrophages, also designed as CCR2<sup>+</sup>CX3CR1<sup>-</sup>

cells, exhibit a typical inflammatory phenotype and secrete high levels of pro-inflammatory cytokines including interleukin (IL)-6 and IL-12. Conversely, M2 macrophages or  $CCR2^{-}CX3CR1^{+}$  cells exhibit anti-inflammatory and tissue repair function and proficiently produce high amount of anti-inflammatory cytokines such as TGF- $\beta$ 1 and IL-10 (Sica and Mantovani, 2012; Biswas and Mantovani, 2010; Gordon and Taylor, 2005).

Interestingly, a more detailed analysis of TGF- $\beta$ 1<sup>+</sup> macrophages in HD revealed phenotypic heterogeneity of these cells at different stages of the disease. In particular, we found that a large number of inflammatory M1 macrophages dominated the early stage of the disease at the expenses of M2 macrophages; a phenomenon that was reversed later, in symptomatic HD patients.

Moreover, analysis of the percentage of either IL-12 or IL-10-producing cells further support macrophages heterogeneity in HD. IL-12<sup>+</sup> macrophages were significantly more numerous in preHD subjects than they were in symptomatic HD patients, who instead showed a remarkable increase of percentage of IL-10<sup>+</sup> macrophages. Changes in the percentage of both cytokines producing cells would explain the disease stage-dependent variation of cytokines levels in HD individuals (Björkqvist et al., 2008). While increased percentage of IL-12<sup>+</sup> macrophages (M1) may explain the inflammation state in the early stage of the disease, elevated IL-10 producing macrophages (M2) in advanced disease stage may enhance an adaptive immune response, convey neuroprotective signals and, possibly, outline a potential repairing attempt of tissues in HD. Yet, the mechanisms that govern macrophage polarization to different phenotypes remain to be defined, however, our results suggest that it could be mediated by a variable activity of NF- $\kappa$ B. NF- $\kappa$ B is widely known for its role in the regulation of inflammation and immune response (Mattson and Camandola, 2001). It is an inducible heteromeric transcription factor classically composed of p50 and p65 subunits. While p65 possesses a transactivation domain and modulates most of the NF- $\kappa$ B's transcriptional activity, p50 does not (Hu et al., 2004; Driessler et al., 2004).

Activation of NF- $\kappa$ B promotes M1 macrophage polarization and turns inflammation processes on (Sica and Bronte, 2007), while its inhibition results in switching the inflammation processes off (Liu et al., 1999; Sica et al., 2000; Porta et al., 2009). Dysregulation of NF- $\kappa$ B activity has been implicated in the pathogenesis of multiple diseases such as inflammatory diseases and neurodegenerative disorders (Liu and Malik, 2006) including HD (Khoshnan et al., 2004; Hsiao et al., 2013).

Interestingly, in the present study we found that NF- $\kappa$ B pathway changed along the disease course. Unlikely a relatively high expression of NF- $\kappa$ B -p65 early in the disease, HD patients showed levels of NF- $\kappa$ B -p65 reduced when compared to preHD subjects and similar to healthy controls. Whether the reduction of NF- $\kappa$ B -p65 in HD patients is due to its selective degradation (Reijonen et al., 2010) or depends on anti-inflammatory strategy that macrophages can adopt to counteract the overproduction of inflammatory cytokines needs to be further investigated.

Predominance of classical NF- $\kappa$ B heterodimer p50/p65 promotes M1 polarization, whereas M2 polarization is selectively mediated by p50/p50 homodimers (Porta et al., 2009). NF- $\kappa$ B-p50 plays a crucial role in the control of M1-vs. M2-driven inflammation by selectively promoting the production of the anti-inflammatory cytokine IL-10 (Cao et al., 2006) that, in turns promotes the formation of p50/p50 homodimer (Grütz, 2005) and inhibits NF- $\kappa$ B activity (Driessler et al., 2004). Based on this evidence, we hypothesized that the lower availability of NF- $\kappa$ B-p65 late in the disease could correlate with a preferential formation of p50/p50 homodimers thereby driving anti-inflammatory IL-10 gene transcription and subsequently favoring M1-M2 switch (Fig. 12).

Although little is known about the acquisition and maintenance of M2 phenotype, we believe that M1-M2 switch in HD, in all probability, points out differential roles of macrophages in the disease induction or progression and may provide protection against overwhelming uncontrolled inflammation.

An additional important point of this study is that, similarly to the periphery, the ability of cells to produce TGF- $\beta$ 1 in the brain varied in a disease stage-dependent manner, indicative of a possible link between peripheral dysfunction and central defects. TGF- $\beta$ 1 immunoreactivity was merely detectable in the pathological grade I HD brains and increased dramatically with the severity of pathological grades. Increased HD brain expression of TGF- $\beta$ 1, in advanced pathological grades, correlated with increased GFAP immunoreactivity, indicating a spread reactive gliosis, a coordinated cellular response usually aimed at mitigating damage to nearby neurons (Bruno et al., 1998; McBride et al., 2003). A phenomenon that could be compared to what occurs in periphery, where anti-inflammatory pattern dominates the late clinical stage of the disease. Since the biological effects of TGF- $\beta$ 1 are diverse, the pathological significance of both clinical stagedependent changes of TGF- $\beta$ 1 content in periphery and pathological grade-dependent changes in post-mortem brain tissues of HD patients is thought to be complex and, further studies are needed to specifically address this issue.

A crucial finding of our study is that changes of number of TGF- $\beta$ 1<sup>+</sup> macrophages correlated with HD clinical features. This observation strengthens the hypothesis of peripheral TGF- $\beta$ 1 as a potentially valid parameter for monitoring disease development. Collectively, this study suggests that alteration in monocytes/macrophages homeostasis plays a critical role in establishing the defective production of TGF- $\beta$ 1 in HD and describes an interesting parallelism between peripheral dysfunction and central defects. We believe that the discovery of macrophages plasticity and an unbalanced M1/M2 phenotype in HD point out a novel biological process that besides elucidating the issue of TGF- $\beta$ 1 production, could explain the variable inflammatory profile in HD and eventually define the possible molecular mechanism underlying immune response in the disease. Macrophages heterogeneity in HD likely reflects dynamic variation in the micro-environmental changes during the transition from early HD stage to advanced HD stages, which would result in progressive modulation of the NF- $\kappa$ B activity in macrophages and their subsequent conversion from an M1 to an M2 phenotype. To our knowledge, this is the first evidence of a biological phenomenon never described before in HD. Understanding the biological mechanisms whereby each of the macrophages subset is induced to assume these different roles may provide new opportunities to therapeutically manipulate immune response in HD. In conclusion, we believe that our study may be of substantial clinical relevance, as it may lead to the identification of the early stage of HD. The identification of markers for predicting HD onset would advance the design of clinical trials to delay onset or slow progression in HD.

***LEGENDS AND FIGURES***

**Figure 2: Total number of whole blood cells is similar between HD individuals and healthy controls.** (A) Representative multicolour flow cytometry light-scatter plot of whole blood samples. Lymphocytes, monocytes and granulocytes were distinguished from each other on the basis of physical parameters by using forward (FSC-H, cell volume index) and side light-scatter patterns (SSC-H, cell density index). (B) lymphocytes, (C) granulocytes and (D) monocytes in HD individuals (n= 81, gray bars) and healthy control subjects (n=26, white bar) was calculated by FACS analysis. Cells number is expressed as  $n^{\circ}$  cells  $\times 10^3$ /ml. Data are shown as mean  $\pm$  s.d.

**Figure 3: The percentage of lymphocytes and granulocytes producing TGF- $\beta$ 1 does not vary between HD individuals and healthy control subjects.** Percentage of TGF- $\beta$ 1 producing lymphocytes (A) and granulocytes (B) in HD (gray bars) individuals (n= 81) and healthy control (white bar) subjects (n=26). TGF- $\beta$ 1-producing cells were identified in FL-2<sup>+</sup> fluorescence scatter. Positive staining for TGF- $\beta$ 1 was represented as the percentage of the curve exceeding the fluorescence value at 95% of the isotype control curve. Data are shown as mean  $\pm$  s.d.

**Figure 4: Percentage of TGF- $\beta$ 1+ monocytes is reduced in presymptomatic (preHD) and stage I HD individuals.** A and B, Percentage of TGF- $\beta$ 1+ cells and relative Mean Fluorescent Unit (MFU) in HD individuals (gray bars) and healthy control subjects (white bar) by FACS analysis. Data are shown as mean  $\pm$  s.d. \*\* p<0,001, \*\*\* p<0,0001 (Student's t-test)

**Figure 5: Percentage of TGF- $\beta$ 1<sup>+</sup> macrophages is reduced in pre-manifested subjects (preHD) and stage I HD patients.** A, Representative multicolour flow cytometric analysis of TGF- $\beta$ 1<sup>+</sup> macrophages for each group: control subject (CTRL), pre-manifested subjects (preHD) and stage II HD patients (HD). TGF- $\beta$ 1<sup>+</sup> cells were identified in FL-2<sup>+</sup> fluorescence scatter (black histogram). Isotype control (gray histogram) was used to determine the 95% confidence interval of nonspecific fluorescence. B and C, Percentage of TGF- $\beta$ 1<sup>+</sup> cells and relative Mean Fluorescent Unit (MFU) in HD individuals (gray bars) and healthy control subjects (white bar) by FACS analysis. Data are shown as mean  $\pm$  s.d. \*\* p<0,001, \*\*\* p<0,0001 (Student's t-test). D, Analysis of TGF- $\beta$ 1 gene expression in preHD subjects, stage II

HD patients and healthy controls. Data are shown as mean  $\pm$  s.d. \* $p < 0,05$ , \*\*  $p < 0,001$ , \*\*\* $p < 0,0001$  (Mann Whitney non parametric test).

**Figure 6: Macrophages display distinct phenotypes during disease stages in HD.** A and B, Percentage of CCR2<sup>+</sup>CX3CR1<sup>-</sup> and CCR2<sup>-</sup>CX3CR1<sup>+</sup> macrophages in pre-manifested subjects (preHD), severe HD patients (HD) and healthy control subjects (CTRL) by FACS analysis. CCR2<sup>+/+</sup>CX3CR1<sup>+/+</sup> cells were identified in FL1<sup>+/+</sup>-FL2<sup>+/+</sup> fluorescence scatter, respectively. Data are shown as mean  $\pm$  s.d. \*  $p < 0,05$ , \*\* $p < 0,01$  (Student's t-test).

**Figure 7: Percentage of IL-12 and IL-10-producing macrophages changes during HD in accordance with macrophage phenotypes.** A and B, Percentage of IL-12 and IL-10 positive cells in premanifested subjects (preHD), severe HD patients (HD) and healthy control subjects (CTRL) by FACS analysis. Data are shown as mean  $\pm$  s.d. \* $p < 0,05$  (Student's t-test).

**Figure 8: NF- $\kappa$ B -p65 protein expression is increased in macrophages from preHD subjects.** A, Representative immunoblot and densitometric analysis of NF- $\kappa$ B -p65 expression in healthy controls, pre-manifested subjects (preHD) and severe HD patients (HD). Graph shows the densitometric analysis performed on four individuals per group. Bars represent the mean values  $\pm$  s.d. \* $p < 0,05$  (Student's t-test).

**Figure 9: Expression of TGF- $\beta$ 1 in post-mortem brain increased from pathological grade II to grade IV in HD patients.** Representative microphotographs of immunohistochemical analysis for TGF- $\beta$ 1 expression on formalin-fixed and paraffin-embedded post-mortem striatal tissues from control subjects and HD patients at different disease stages.

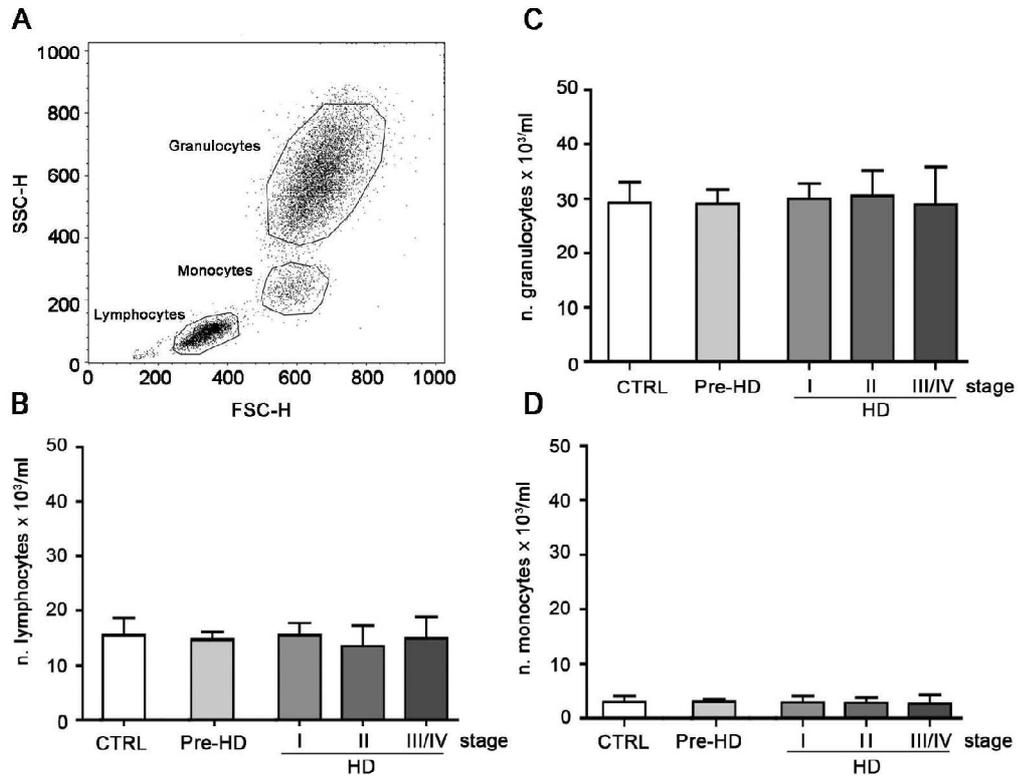
**Figure 10: Increased TGF- $\beta$ 1 expression in HD human post-mortem brain samples correlates with activation of glial cells during disease progression.** Glial activation was determined by glial fibrillary acidic protein (GFAP) immunoreactivity. Representative microphotographs of double fluorescent staining for TGF- $\beta$ 1 and GFAP

in post-mortem striatal tissues of control subjects, and HD patients at different pathological grades (from II to IV).

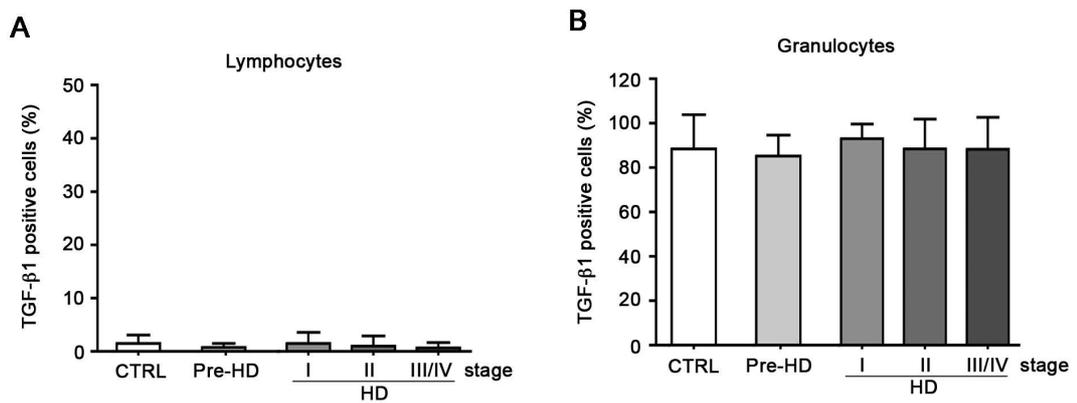
**Figure 11: Percentage of TGF- $\beta$ 1+ macrophages correlates with clinical and genetic parameters in HD individuals.** Linear regression analysis yields a positive correlation between percentage of TGF-  $\beta$ 1+ cells and age at onset (A; n=69), disease burden (B; n=76), HD development index (C; n=79) and UHDRS1 (D; n=72). Percentage of TGF- $\beta$ 1<sup>+</sup> macrophages negatively correlates with disability scale (E; n=74), UHDRS4 (F; n=70), UHDRS2 (G; n=64), MMSE (H; n=67) and UHDRS3 (I; n=72). Each dot represents a single subject.

**Figure 12: Differential polarization of macrophages during HD.** Schematic diagram showing the classically (M1), or alternatively (M2) activated macrophages with their cell-specific cytokine production in HD. It is proposed that switching of macrophages polarization, from M1 to M2, is paralleled by a potential dysregulation of NF- $\kappa$ B activity along HD course. M1 macrophages, also designed as CCR2<sup>+</sup> cells, dominate the early stage of the disease and participate in proinflammatory activities. M1 polarization may be mediated by mutant Htt (mHtt) that up regulates NF- $\kappa$ B activity (higher levels of p65) and promotes pro-inflammatory cytokines (IL-12, IL-6) production (left). Conversely, a preferential M2 polarization prevails late in the disease. M2 macrophages, also identified as CX3CR1<sup>+</sup> cells orchestrate anti-inflammatory activity by releasing high levels of anti-inflammatory cytokines and growth factors mainly involved in the immunosuppression and tissues repair. Decreased activity of NF- $\kappa$ B and concurrent over-expression of p50 homodimer are likely to represent putative biological events driving M2 polarization in HD (right).

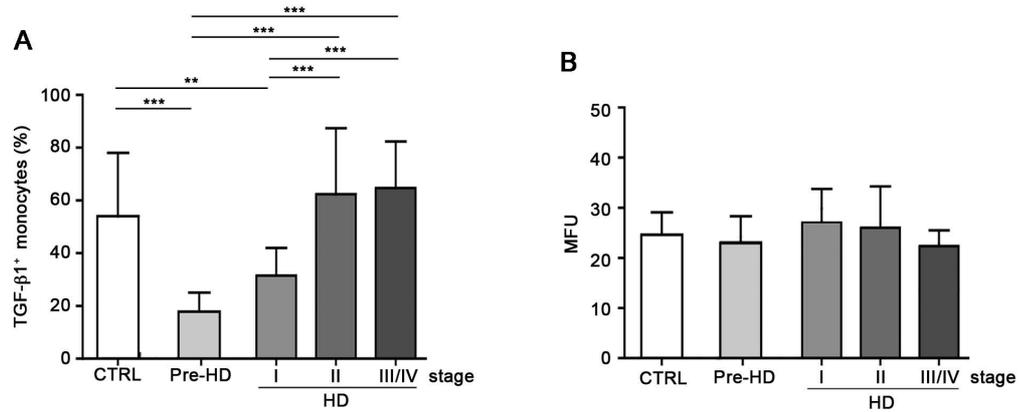
**Figure 2**



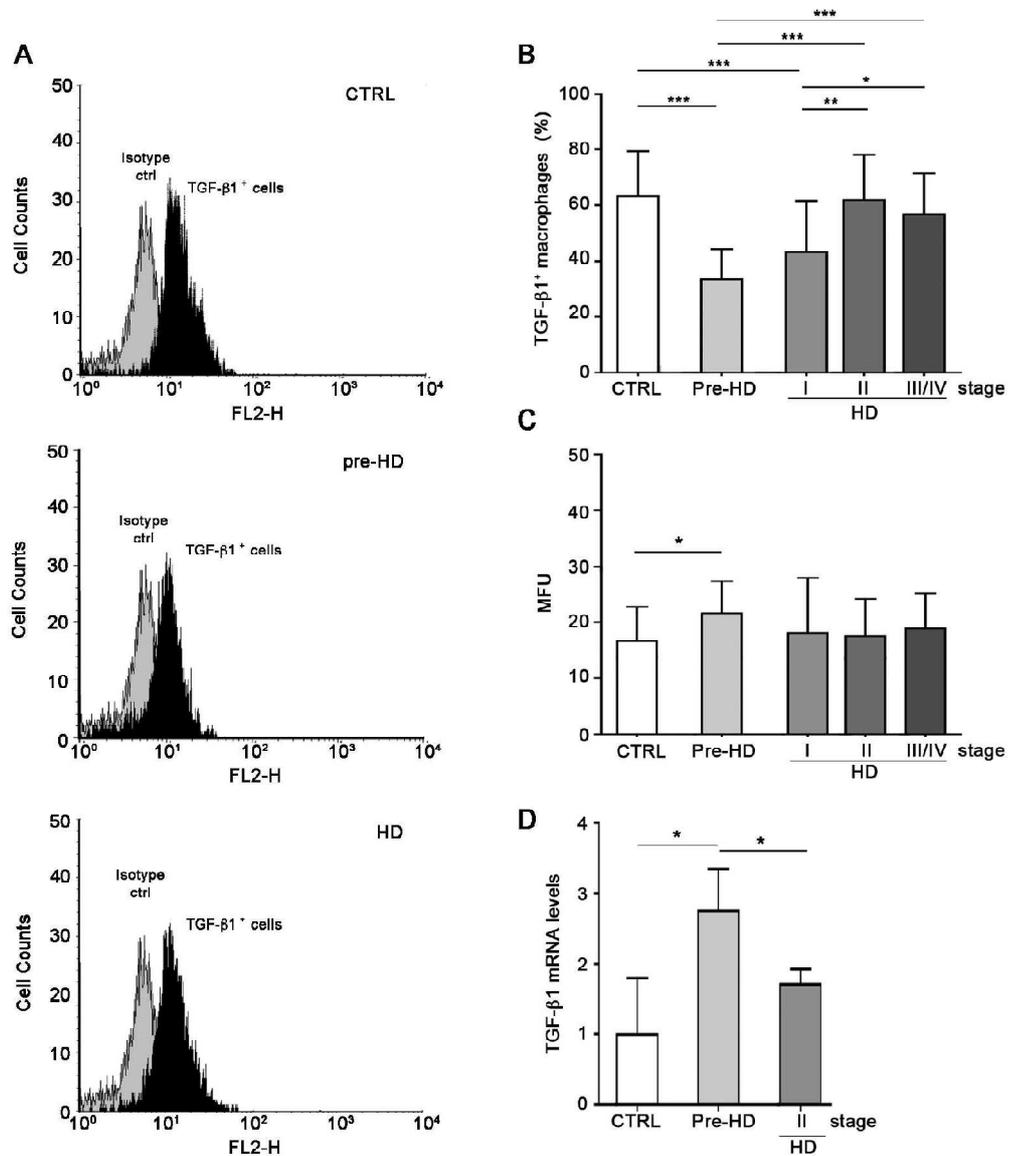
**Figure 3**



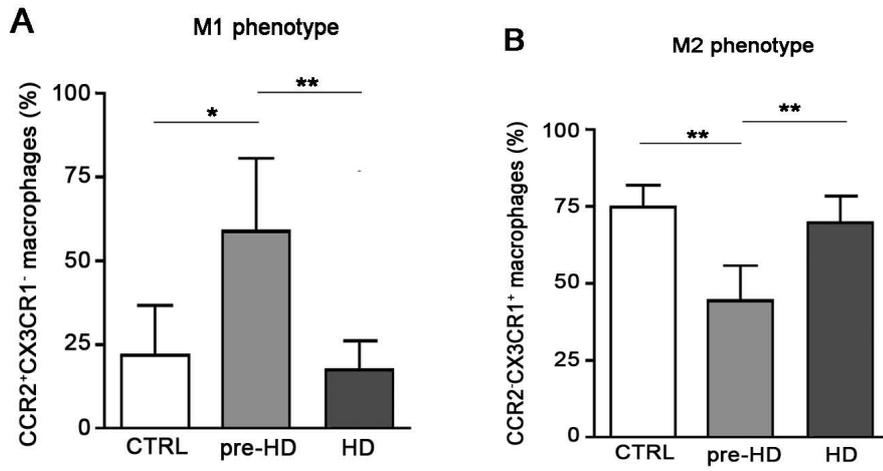
**Figure 4**



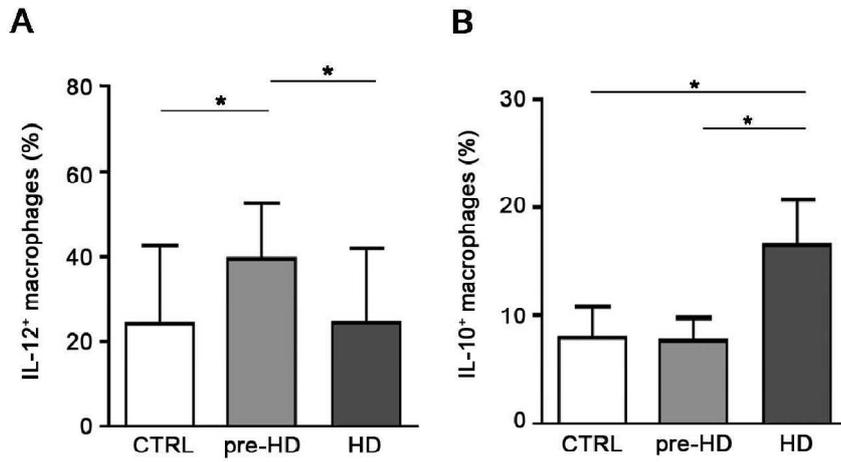
**Figure 5**



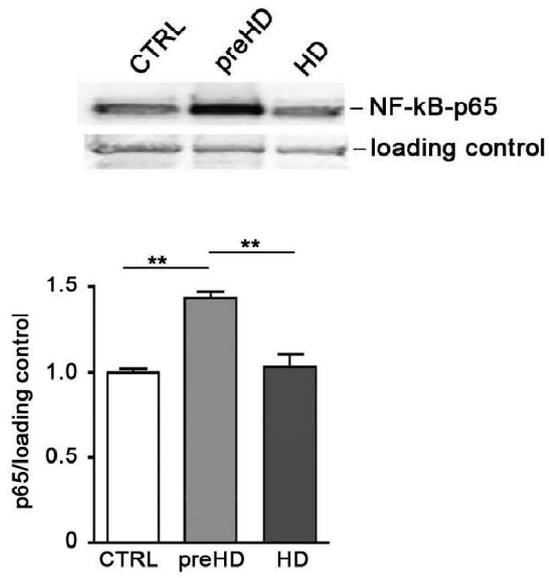
**Figure 6**



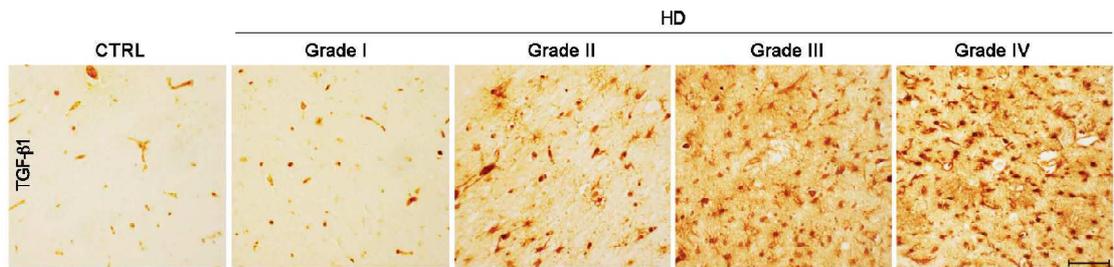
**Figure 7**



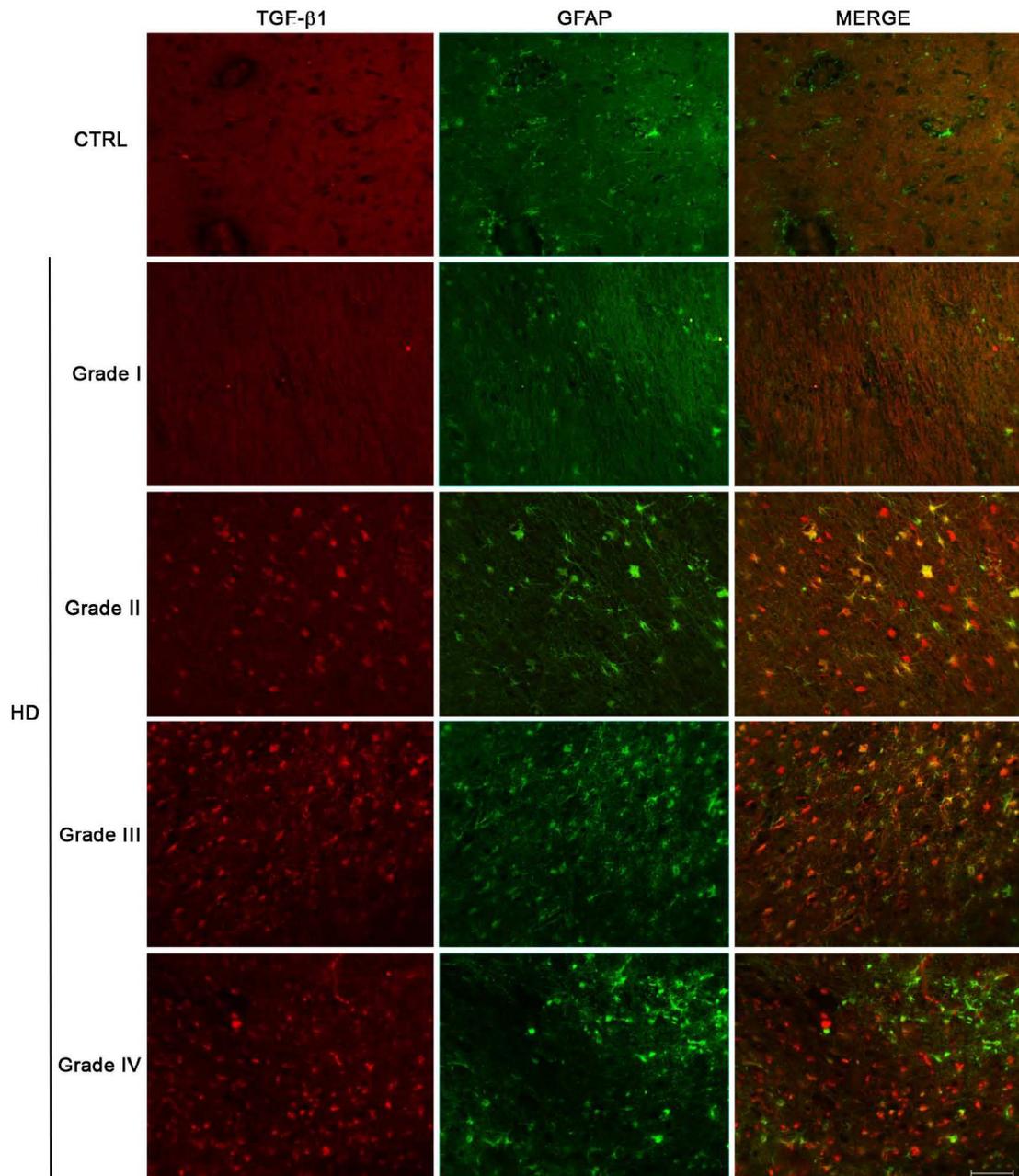
**Figure 8**



**Figure 9**



**Figure 10**



**Figure 11**

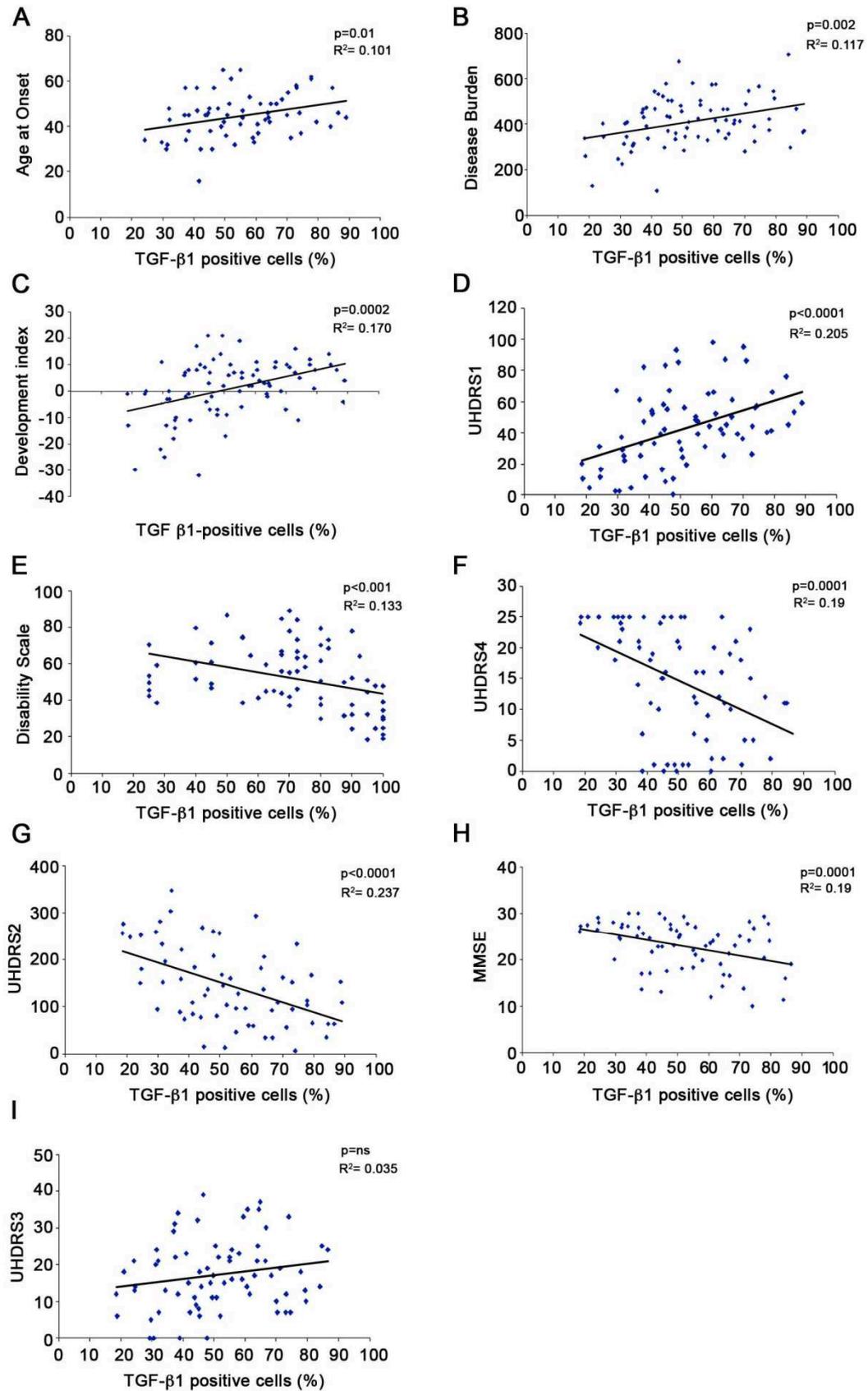
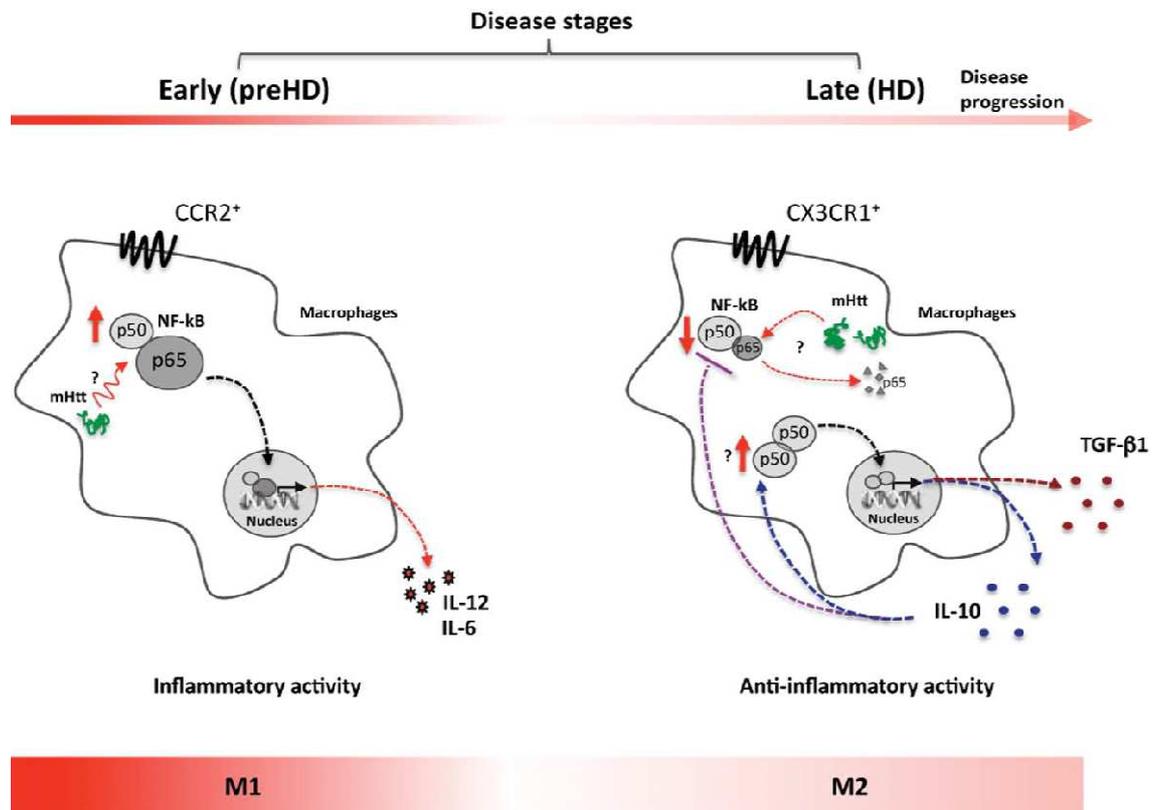


Figure 12



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