Blood and Biomarkers in Huntington's Disease

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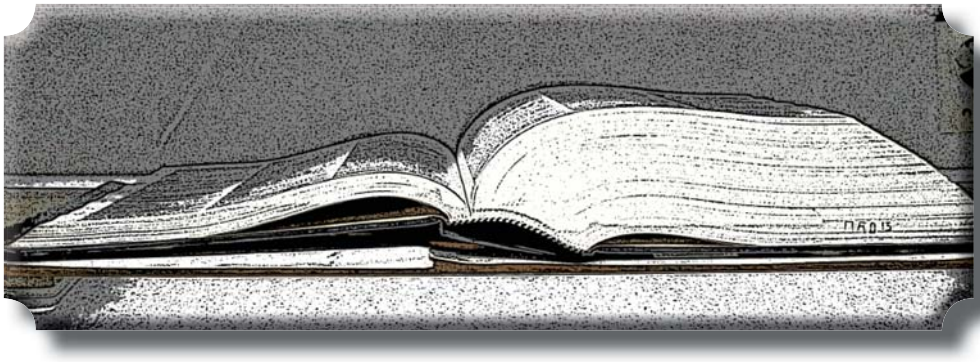
Chapter 1

General Introduction



1.1 Huntington's Disease

1.1.1 Huntington's disease discovery and epidemiology

Huntington's disease is an autosomal dominant neurodegenerative disease that manifests through motor and psychiatric symptoms. Huntington's disease was first described and named after George Huntington in 1872; who in his paper "On Chorea" described the major aspects of the disease [1]. However, it was not until 1983 when the disease was first mapped to a restriction fragment length polymorphism marker (RFLP) (D4S10/G8) [2] on chromosome 4 and it took 10 years more (1993) before it was linked to "interesting transcript 15" (IT15) following which the HD gene was identified and isolated [3]. Since current knowledge about the human genome was unavailable at the time, the long search for the identification of the disease gene was further limited by the telomeric location of the RFLP marker, the physical distance as well as uncertain recombination frequencies between the marker and the disease locus [4,5]. HD population prevalence exhibits significant geographic differences depending on local, past migration patterns and ethnicity. The disease is most prominent in Western European populations with a rate of occurrence of 5-10 in 100.000, a prevalence of approximately 1 in 10.000 in Caucasian populations [6,7] and is least frequent in Japanese, Chinese, Finnish and African descent individuals [8]. However, as suggested by Evans et al., the disease prevalence has risen significantly in the last decades and it was found that for UK the number of HD patients was 12.3 per 100.000 [9]. Localized geographic areas exhibit very low as well as very high prevalence with the most notable example being the population of Lake Maracaibo in Venezuela which was also originally used to identify the disease gene [10].

1.1.2 Huntington's disease genetics

Huntington's disease is caused by an expanded CAG repeat in the HTT gene. Individuals that carry a CAG repeat higher than 35 are the ones that will most likely develop the disease symptoms at some point during their lifetime [11]. People with 36-39 repeats are known to carry a reduced penetrance allele and they may develop the disease rather later in life (40% chance asymptomatic at age 65) [12]. For most mutation carriers disease

onset takes place between 35 and 50 years old [13]. However, symptom onset can occur at any time during the carrier's lifetime and is inversely correlated to the CAG expansion length. As a result, people with 40 repeats or more will develop the disease and when the repeat exceeds 50 symptoms may develop as early as childhood. The elongated CAG tract results in a mutant huntingtin protein with an elongated polyglutamine tract that is associated with protein aggregation and toxic gain-of-function properties [14]. During meiotic transmission, the HD repeat is unstable in more than 80 % of the cases (increases and decreases) while the largest increases are seen in paternal transmissions [15]. This meiotic instability leads to genetic anticipation of the disorder (increasing intergenerational disease severity) but also to the generation of de-novo HD mutation carriers from unaffected parents; especially from parents which exhibited an increased glutamine repeat number and within the intermediate repeat area of the disease (27-35 CAGs) [16,17]. Nevertheless, the HD gene mutation only explains about 50-77 % of the variance in the age of symptom onset and scientists are continuously looking for genetic as well as environmental modifiers of disease onset [18,19]. Several genetic modifiers have been identified that further influence the trinucleotide repeats instability as well as the age of onset of the disease. Such genetic modifiers influence the manifestation of disease phenotypes and partially explain the variability and degree to which these symptoms develop [18,20,21]. Apart from genetic modifiers, there are also specific factors that lead to the unstable transmission of the, disease causing, trinucleotide repeat expansion and include toxic oxidation which is known to cause progressive age-dependent expansions [22,23] as well as population specific haplotypes made up of sets of single nucleotide polymorphisms [24]. On the other hand, genetic modifiers that influence the disease onset include functional polymorphisms in key genes involved in neuronal processes, the processing of huntingtin itself, gene transcription but also metabolism. Examples of affected genes include the a) kainate receptor *GluR6* [25] b) glutamate receptor, ionotropic, N-methyl-d-aspartate – *GRIN2A/2B* [26] c) gene transcription factor *TP53* [27] d) the Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha (*PPARGC1A*) transcription factor that is involved in regulating energy metabolism genes [28,29]. Finally, in a recent very large genome wide association study two different chromosomal loci were discovered (chromosomes 8 and 15) that exhibited independent effects of disease onset acceleration and/or delay [30]. The functional variants within

the above two chromosomal loci are thought to affect genes involved in DNA synthesis and repair, mitochondrial energetics and oxidative stress (*FAN1, MTMR10, RRM2B, UBR5*). The presence of all the above genetic differences in HD mutation carriers shows that detailed genotypic information will be required in order to efficiently address the manifested disease symptom variability.

1.1.3 Huntington's disease motor symptoms

One of the most prominent features of HD is the movement disorder known as chorea [31]. Chorea is characterized by hyperkinetic dance like movements and ranges from random, exaggerated gestures and expressions to continuous and violent movements. Huntington's disease patients are also characterized by non-choreatic, hypokinetic movements such as bradykinesia and dystonia. Hypokinetic movements are usually more prominent during middle and late stages of the disease while, on the other hand, hyperkinetic movements are usually present at early to middle stages of the disease. Specifically, as the functional capacity of patients declines, the presence of chorea lessens and the presence of dystonia is intensified [32]. Additional motor symptoms include the slowness of movements (bradykinesia), rigidity and clumsiness. Aside from chroreatic or dystonic motor symptoms patients also exhibit skeletal muscle wasting and weight loss (see 1.2.4) [33]. The above two observations take place irrespective of the fact that HD patients receive a steady caloric intake [34]. HD patients further exhibit ocular abnormalities such as the inability to suppress reflexive saccades to a new optical stimulus, and are characterized by delayed initiation of voluntary saccades (reflexive and voluntary eye control respectively) [35]. These ocular dysfunctions can be in turn linked to potentially affected brain areas since it has been shown that saccadic eye movement control is partially controlled by the hippocampus, the inferior parietal lobule and the prefrontal cortex [36,37]. Finally, in subsequent and more advanced stages of the disease the patients are exhibiting dysarthria (speech problems), dysphagia (swallowing difficulties) and balance disturbances, with the latter two problems constituting potential death causes by aspiration pneumonia or serious injury respectively [38-41].

1.1.4 Huntington's disease cognitive and psychiatric symptoms

HD is characterized by cognitive and psychiatric symptoms such as depression, irritability, apathy, anxiety and dementia. The above symptoms are usually evident well before the onset of any motor symptoms and get significantly worse over time. Nevertheless, psychiatric symptoms do not always correlate with cognitive and motor aspects of the disease and specific symptoms are sometimes more highly associated with certain disease stages, such as the manifestation of depression at the same time or after onset of motor symptoms [42,43]. Previous studies have identified that depression is a significant component of the prognostic signature of HD and that such affective disorders can appear as early as 20 years before choreatic and cognitive symptom onset [44]. Another study of depression in HD has reported that over 40 % percent of HD patients had significant symptoms of depression and over 50 % had previously received treatment for depression [45]. These numbers are significantly higher than the prevalence of depression in the general population (8-12 %) [46]. The exact reason behind the high number of reports of depression in HD is not clear. However, it has been hypothesized that apart from potential perturbed biological pathways this could be also due to the adjustment of these patients to the idea that they suffer from a disabling, terminally ill disorder [47,48]. Moreover, anxiety and irritability have also been reported in HD at a prevalence varying from 34-61% and 38-73% respectively [49] which is also much higher than the reported lifetime prevalence of any other anxiety disorder as reported from the World Mental Health surveys[50]. HD patients are likewise often characterized by apathy and specifically reduced energy and lack of drive. It has been proposed that in HD and other neuropsychiatric diseases, apathy is distinct from depression and associated with disinhibition and aberrant motor behavior, in contrast to anxiety and irritability which characterize depression [51]. Finally, obsessive and compulsive symptoms as well as psychotic symptoms have also been reported in HD mutation carriers, even though at a much lower prevalence (10-52 % and 3-11% respectively) [43,49].

1.2 Huntington's disease neuropathology

Huntington's disease is characterized by atrophy of the cerebral cortex and the striatum

and HD patients are known to lose up to 40% of their brain volume compared to healthy individuals [52,53]. Neurodegeneration affects mostly the medium spiny projection neurons of the striatum while the interneurons are relatively spared [54]. The neurodegeneration mechanism of the mutant huntingtin protein however appears to be complex and neuronal death does not always correlate to huntingtin nuclear inclusions [55]. One of the most used methods to classify HD neuropathological severity is the Vonsattel ascending severity order grading system (Grades 0-4) that was formulated using postmortem brain specimens in order to identify the microscopic and macroscopic changes taking place in mutation carrier brain tissue [56]. Vonsattel et al. reported a caudate nucleus neuronal loss of 50-95% from grades 1-4 and a big increase of astrocytic population during grades 2-4. The authors also reported that the earliest changes were found in the medial paraventricular areas of the caudate nucleus and dorsal part of the putamen as well as the fact that anatomical changes were usually lagging behind the clinical symptoms something that has also been confirmed by later studies [57,58]. Apart from the neuronal degeneration pattern that is evident in the striatum of HD patients, other areas are affected too such as the substantia nigra, globus pallidus, thalamus and cerebellum [59]. The multiplicity and the variable degree of degeneration of the affected brain areas constitute the basis for the diverse clinical phenotype and disease progression rates the patients exhibit. The latter assumption has been supported by imaging studies, which have revealed a widespread brain tissue degeneration and significant volume reductions in almost all brain areas; occurring even in early stage patients [60-63].

1.2.1 Huntingtin protein structure and function

Wild type Huntingtin (Htt) protein is 348 kDa, expressed ubiquitously and its highest levels are found in the brain and the testis [3,64-66]. The exact 3D and molecular structure of the protein has not been yet achieved due to the high molecular weight of the protein and the problems this causes in X-ray crystallography. Currently, in an ongoing attempt to identify its protein structure huntingtin has been transferred to the international space station in an effort to take advantage of extraterrestrial weightlessness condition and microgravity crystallization techniques (NASA mission experiment CASIS PCG HDPCG-1). Structurally, the HD protein is characterized by a CAG repeat and

by a proline rich domain which are located in the protein N terminus [3]. The presence of similar homopolymeric glutamine and proline stretches in many transcription factors has lead scientists to the conclusion that huntingtin is also one such protein with similar functional properties [67-69]. Apart from its distinctive N-terminal structure the protein possesses several well described consensus motifs that are required for many protein-protein interactions – which is another characteristic of Htt [70]. Such patterns, sequences or motifs include: a) Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1 - (HEAT) - repeats [71] b) proteolytic cleavage sites that allow the fragmentation of the protein through the function of caspases 2,3,6 and 7 [72,73] c) nuclear localization sequences [74] and d) posttranslational modification sites such as those for its phosphorylation and ubiquitination [75-78]. The wild type protein was initially considered to be found only in the cytoplasm and associated with the Golgi apparatus, the microtubules and the endoplasmic reticulum [79-81]. However, an early study by Hoogeveen et al. and later one by De Rooij et al. showed that huntingtin was also present in the nucleus [82,83]. This discovery further emphasized important protein roles such as transcriptional control, a role supported by the structural features and motifs that Htt possesses, like poluglutamine stretches and leucine zipper motifs. Huntingtin is also found in clathrin coated vesicles and it has been proposed that it interacts with proteins involved in cytoskeletal transport, receptor mediated endocytosis as well synaptic recycling [84,85]. Even though the highest huntingtin levels are found in the central nervous systems [86-88] the exact function of the wild type protein is still not completely understood. Nonetheless early research in the field into the function of the protein has shown that huntingtin is required during gastrulation in early embryogenesis and has a vital role in the functioning of the basal ganglia and neuronal survival [89,90]. Additionally, further studies have demonstrated that huntingtin may possess many further roles in many key cellular pathways. Several roles have been proposed for huntingtin: possessing an anti-apoptotic role and protecting from excitotoxicity [91,92], regulating the production of neurotrophic factors such as brain derived neurotrophic factor (BDNF) [93,94], participating in or disrupting axonal transport and vesicle trafficking [95,96] and regulating synaptic function through partner proteins such as PSD-95 [97,98].

1.2.2 Mutant huntingtin aggregates and inclusions

Soon after the discovery of the HD causal mutation the presence of neuronal aggregates and inclusions of mutant huntingtin was also established as one of the main disease pathological hallmarks. The designation of the aggregates as the main disease hallmark was made possible through a series of successful experiments that used mouse and human studies to initially locate neuronal intranuclear inclusions and dystrophic neurites in HD brain [99] and later identify their role in toxicity, or protection therefrom, and in disease neurological phenotypes [100,101]. Even though the mechanisms through which Htt inclusions or Htt fragment inclusions lead to toxicity and neurodegeneration have not been completely elucidated. Some of the hypotheses advanced include interference and sequestration of essential cellular proteins and transcription factors, proteasomal component interference and functional impairment [102,103], apoptosis [104], mitochondrial function damage [104,105] and alterations in cytoskeletal and synaptic function [106]. The factors that determine the capacity of the mutant Htt to aggregate include the length of the polyglutamine expansion and the length of huntingtin polyglutamine fragments [107], the specific conformation of the mutant protein as well as the sequences that flank the polyglutamine stretch such as the poly-proline motif located downstream of the CAGs [108]. Miller et al. have suggested that the rate-limiting step for the formation of inclusion bodies (nuclear or cytoplasmic aggregates) are the different conformational changes in monomeric Htt exon 1 fragments [109]. This suggests that only modest reductions in the levels of the mutant proteins or even their relative conformation ratios can have a profound effect in aggregate formation and toxicity [109,110]. Another significant factor correlated with the increased aggregate formation is the mutant protein proteolytic cleavage and the subsequent formation of N-terminal fragments. Specifically, the function of proteases such as caspase and calpain have been shown to increase the formation of aggregates and neuronal susceptibility to cell death [111-113]. Independently of all the above factors influencing aggregate formation, the propensity of the mutant huntingtin to aggregate and cause disease pathology could additionally depend on chaperones [114,115], posttranslational modification enzymes [116,117] and can be influenced by the specific neuronal cell type properties and the mutant huntingtin expression levels and dynamics in particular cell types such as primary

neurons, astrocytes, oligodendrocytes and microglia [118-120].

1.2.3 Huntington's disease brain pathology

Postmortem examinations of HD carriers have shown that the brain tissue changes are mostly evident in advanced stages [121] and are characterized by tissue losses in the cerebral cortex and thalamus (reductions 21-29%) as well as the caudate and putamen (57% and 64% tissue loss respectively). Even though these large tissue losses are more visible in later disease stages, recent studies have shown that such brain atrophy and white matter disorganization events can even take place before the onset of disease clinical symptoms [122]. For example, medium spiny neurons make up to 95% of the striatal neuronal cell population and contain a plethora of metabotropic, glutamate and dopamine receptors [123,124]. These receptors have been the subject of intense research as to the possible mechanisms through which they can cause or contribute to neuronal cell death (see 1.3.4). In the majority of HD cases the binding capacities of these receptors is reduced and this capacity reduction has been correlated to neuronal cell death [125,126]. Nonetheless it is not completely clear yet if this receptor dysfunction leads to cell death or vice versa [126]. On the other hand, some cell types such as the striatal interneurons, the medium-sized calretinin-positive interneurons and the neuropeptide Y/somatostatin containing neurons are relatively spared, even in more advanced disease stages [127]. The reason that these cell types are less susceptible to the disease is not yet fully understood. It has been postulated however that this preservation can be related to the distribution of the cells in the corresponding brain areas as well as their relative position to excitatory neuroreceptors responsible for cell over-excitation and subsequent cell death [128]. Finally, it has been shown that the cellular and neurochemical changes that take place in HD patient brains are accompanied by gliosis [129-131]. Gliosis is characterized as an increase in the population as well as the activation of astrocytes, microglia and oligodendrocytes [128]. Microglial activation has been found widespread through the cortex, the pallidum and striatum and it has been even found to be present in pre-symptomatic carriers [132,133].

1.2.4 Huntington's disease peripheral pathology

Even though Huntington's disease has been traditionally considered as a brain disorder it is characterized by additional peripheral symptoms such as weight loss and skeletal muscle wasting, glucose intolerance, heart and gastrointestinal problems as well as testicular atrophy [134-138]. An increasing number of studies points to the fact that these peripheral symptoms are not solely or even partially due to neurodegeneration of brain cell structures and brain factors but are a result of the mutant protein expression in the specific peripheral tissue cells and occur in parallel with the central nervous system [107,139]. Notably intracellular mutant protein inclusions as well as peripheral skeletal muscle cell dysfunction have been shown to be present even when the peripheral cell types are isolated [140,141]. Weight loss is one of the most common symptoms and especially in symptomatic HD individuals. Several studies have indicated that this weight loss is not only associated with nervous system damage but is also a result of an increased metabolic rate in these patients [142,143]. Moreover, it has also been shown that weight loss does not always follow symptom onset and a multifactorial system could govern the start of weight changes in symptomatic and pre-symptomatic patients [136] Furthermore according to a different study skeletal muscle wasting can also be attributed to expression of the mutant huntingtin protein in the myocytes and a transition from slow-twitch to fast-twitch muscle fibers [144]. In addition, and using mouse models, it was shown that mutant huntingtin affects gene expression patterns in myocytes and that these expression changes are in parallel with early pathological and behavioral changes [144,145]. Other human as well as mouse studies have shown that HD mutation carriers are more likely to develop osteoporosis, diabetes, pancreatic dysfunction and impaired glucose tolerance [146-148]. Finally, the role of the immune system has been the focus of several studies and it has been shown that in HD blood is characterized by increased concentrations of proinflammatory and chemotactic cytokines such as interleukins 6 and 8, altered inflammatory signaling and a general overactivation [149-151]. This deregulation of the immune system has been attributed to expression of the mutant Htt protein in the immune system cell types affecting cell signaling and cytokine release and migration [152]. Most importantly such an immune system over activation does not have an isolated cell type effect but appears to influence other prominent peripheral degeneration events described

above such as muscle wasting and gene expression deregulation [153,154].

1.3 Huntington's disease mechanisms of cytotoxicity

Even though HD is a monogenic disorder the disease pathology is very multifaceted. The cytotoxic function of the aggregated mutant Htt protein can exert its effect through a plethora of processes as described above (see 1.2.2). In addition, it has been shown that mutant huntingtin cleavage products (especially N-terminal fragments) exacerbate the disease pathology. They are more toxic than full length huntingtin and increase the number of intranuclear inclusions [55,155]. Huntingtin is proteolytically cleaved by caspases like caspase 3 and 6 and it has been shown that inhibiting caspase activity can reduce toxicity and aggregate formation (see 1.4.5). It has been proposed that increased caspase activation and substrate cleavage, caused in HD by intranuclear Htt accumulation and release of mitochondrial cytochrome c [156], can also be the cause behind phenotypic similarities across other neurodegenerative diseases such as Alzheimer's disease [55,157,158]. In the following paragraphs, previously mentioned and additional mechanisms of cytotoxicity are described in more detail.

1.3.1 Proteasomal deregulation

The ubiquitin-proteasomal system is one of the main mechanisms of cellular protein degradation in the nervous system and is involved in processes such as neuronal transmission and neuronal plasticity [159]. In HD, it has been shown both in humans and animal models that the mutant huntingtin protein is targeted for proteolysis but is resistant to its processing [99]. Studies that used HTT exon1 transfection polyglutamine aggregates labelled with antibodies against parts of the ubiquitin-proteasomal system demonstrated co-localization with the protein inclusions as well as altered localization that could potentially adversely affect the proteasomal system function [160]. The reduced proteasomal ability to cleave the mutant protein as well as other polyglutamine proteins is attributed to the greatly expanded length of glutamine repeats of the mutant protein. As a result, these proteins are getting "stuck" in the 20S subunit of the proteasome and do not allow for the subsequent entry of the remaining catalytic substrates and other

proteins [161]. Additionally, using fluorescence recovery after photo-bleaching (FRAP) optical technology it was shown that the proteasome was irreversibly trapped within the N-terminal mutant huntingtin aggregates both in vivo and in vitro [162]. Even though the results of the previous study did not exclude the possibility of partial proteasome activity or Htt partial degradation – over time this delay in huntingtin or other substrate processing could have deleterious and adverse effects for cellular viability. Moreover, it should be noted that the results concerning the inability of the proteasomal system to degrade the mutant expanded proteins are often contradictory. For example, and in contrast to previous studies, Rousseau et al. have suggested that the aggregation is not a result of the main 20 S proteasomal subunit failure but rather of 19S regulatory particles (Rpt4/Rpt6) flanking the proteasomal catalytic core [163]. Additional data that further diffuse the role of proteasomal dysfunction in HD have shown that it is only the filamentous mutant Htt aggregates that have the potential to inhibit proteasomal function and not the formation of inclusion bodies (IBs) [164]. Despite of the opposing study results described above about the role of proteasome in HD, this proteolytic complex constitutes a very interesting therapeutic target since even if its function is shown not to be affected its activity may be targeted for additional stimulation and enhanced clearance of the polyglutamine fragments [165].

1.3.2 Transcriptional deregulation

The wild type huntingtin protein has also been shown to have a role in transcription regulation and to interact with many other transcription factors and regulatory proteins[166]. On the other hand, during disease it has been confirmed that Htt mutant protein and protein fragment aggregates are involved in the sequestration of proteins involved in transcription, irregular subcellular localization of CAG containing transcripts, alternative splicing; all events that lead to genome wide transcriptional deregulation in HD patients [167]. Specifically, it has been established that important cellular transcription factors such as TATA binding protein, CBP, SP1 and p53 are recruited to these aggregates [168-170]. In two such studies it was shown that Sp1, a well-known wild type Htt interactor, exhibits reduced DNA binding (caused by mutant Htt) and that genes regulated by Sp1 exhibited decreased expression [145,171] . Gene expression microarray

studies in human and animal models have shown that the functional types of those genes whose expression is affected by mutant huntingtin include neuronal structure genes, signaling receptors, axonal receptors and stress response genes [172,173]. In a recent study by Labadorf et al. it was also shown that HD brain deregulated genes were enriched for immune response and neuroinflammatory genes as well as homeotic genes (Hox – Homeobox genes) – a set of genes that has not yet been widely investigated in the light of HD [174]. Furthermore, it has been suggested that these mRNA changes were not attributable to cell loss only and exhibited a distinct disease related brain regional pattern, with gene expression similarities between those expressed in the HD caudate and the motor cortex [175]. Other important factors and regulators that have been implicated in HD transcriptional deregulation and whose function is influenced by the mutant protein include the neuronal factor transcriptional repressor *REST* [176], the peroxisome proliferator-activated receptor gamma (*PPARγ*) [177] and the heat shock protein transcription regulator *NF-Y* [178].

Another major transcriptional mechanism disrupted in HD, which has potentially even greater influence than individual transcriptional factors deregulation, is chromatin remodeling. Chromatin remodeling is regulated by epigenetic processes such as histone posttranslational modifications and DNA methylation [179]. Studies from HD models have reported that mutant huntingtin also interferes with the function of enzymes involved in chromatin remodeling such as histone acetyltransferases, histone methyltransferase, ubiquitin ligases and transglutaminases [180]. Specifically, it has been shown that inhibition of histone deacetylases by inhibitors such us sodium butyrate and phenylbutyrate reversed the mutant protein related neurodegeneration [181,182]. Histone methylation is an epigenetic mechanism used to activate or repress transcription however it has been demonstrated that in HD histone methylation related proteins such as ERG-associated protein with SET domain (ESET) and its activator Sp1 are also deregulated in HD [183] Post-translational modifications as the ones performed by transglutaminases have also been shown to exhibit increased activity while their inhibition with cystamine in HD models ameliorated symptoms and increased the expression of neuroprotective genes[184]. Much attention has been given lately to the role of miRNAs in HD and their gene expression regulatory roles like for miR-9 and the neuronal gene expression RE1-Si-

lencing Transcription Factor (REST) [185,186]. Finally, other ways in which chromatin remodeling is affected in HD and normal function of the histone core proteins is disrupted are monoubiquitilation and post-translation modifications such as phosphorylation of serine, threonine and tyrosine residues [172,187].

1.3.3 Mitochondrial activity and energy metabolism deregulation

Mitochondria are well known regulators of cellular health and are involved in cellular energy production, metabolism as well as apoptosis [188]. The mitochondria are another cellular compartment that is thought to be disrupted in HD and eventually also contributing to neuronal cell death. A lot of studies both in animal models and humans have described many different aspects of mitochondrial dysfunction in HD. The different mechanisms of HD mitochondrial dysfunction can be briefly categorized into : a) mitochondrial membrane destabilization b) reduction of the mobility of the mitochondria c) reactive oxygen species (ROS) overproduction with a concomitant Ca2+ cellular vulnerability through the deregulation of the mitochondrial permeability pore [105]. More specifically, studies have shown that HD mitochondria exhibit increased CAG repeat length correlated membrane depolarization [189], reduced membrane potential requiring lower calcium depolarization loads [190] and reduced CA2+ loading capacity as well as evidence that CA2+ dependent pathways lead to oxidative phosphorylation impairment of HD mitochondria [191]. It has been demonstrated that full length mutant Htt blocks mitochondrial movement and this effect correlates with increased mutant protein expression in the cytosol [192]. The same study showed that the mitochondria accumulated in areas of aggregates while their trafficking was unchanged at neuronal areas that lacked aggregates. Reactive oxygen species, produced by the mitochondria during oxidative phosphorylation, have shown to be increased in HD. This has been attributed to the presence of the mutant protein [193] and microglial over-activation which typically occurs after brain injury or immunological stimuli and has been shown to correlate with disease severity [194,195]. Moreover it has been shown that dysfunctional mitochondrial metabolism in HD patients leads to diminished complex II, III and IV activity in the caudate nucleus [196], decreased glucose metabolism and inhibition

of the metabolic regulator and transcriptional coactivator PGC-1alpha [197-199], as well as increased lactate production in the cortex and the basal ganglia [200,201]. Finally, the deregulation of neurotransmitters such as dopamine and glutamate present at the striatum and their disordered interactions with the mitochondrial system could also account for the preferential vulnerability of these brain areas to the mutant Htt protein which involves but is not limited to processes such as the reduction of cellular ATP stores, deregulation of intracellular Ca2+ levels, increased synaptic dopamine levels and impaired glutamate uptake due to increased production of ROS [202,203].

Despite the fact that so many studies have provided important clues about the deregulation of individual genes and complexes of mitochondrial metabolism it is still unclear to what extend these mitochondrial defects have a causal effect in the disease or if they are just the result of long term disease toxicity [204]. Finally, for any one of the aforementioned genes or molecules to be targeted in therapeutic approaches e.g. to equilibrate their levels, their role in energetic pathways, and their interactors, have to be extensively investigated to avoid unwanted non-specific detrimental effects.

1.3.4 Synaptic dysfunction and excitotoxicity

Several studies have produced strong evidence that HD neuronal cells and especially those that make up the tissues that are first affected in the disease such as the striatum, are especially vulnerable to excitotoxic events and are characterized by aberrant synaptic and extra-synaptic functioning. HD model studies have shown that pre-symptomatic mice exhibit striatal dopamine signaling deficiencies and that dopamine receptor expression gradually declined with age, indicative of the potential role of the dopamine system in HD neuritic malformation and subsequent disease pathology [205,206]. The uptake of another important neurotransmitter, glutamate, has been also shown to be reduced in the prefrontal cortex of HD patients [207]. This reduction in glutamate uptake is usually also accompanied by a reduction in glutamate transporter 1 (*GLT1/EAAT2*) in corticostriatal regions [208,209]. The exact mechanism by which mutant huntingtin interferes with synaptic functioning and the deregulation of neurotransmitter levels is not fully understood yet. Nevertheless, HD model studies have shown that potential mecha-

nisms could include the Htt mutant fragment transcriptional deregulation of glutamate transporters that are mainly expressed by astrocytes that envelop synapses [210,211] and/or an inability to sustain the bioenergetics/glycolytic requirements for the proper functioning of the enzymes responsible for glutamate uptake reactions (GLT-1, GAPDH). These enzymes in turn cause a neurotoxic release of glutamate to the extracellular medium [212,213]. Finally, other complementary studies that have provided clues to the potential mechanisms involved in such excitotoxic cell death have reported that: a) full length mutant huntingtin disturbs neuronal Ca2+ signaling and makes medium spiny neurons sensitive to glutamate-induced apoptotic events [214] b) anti-apoptotic and pro-apoptotic proteins such as the cell death regulator Bcl-2 and the transcription factor p53 are selectively deregulated in striatal neurons and make these neurons more vulnerable to excitotoxic cell death [215] c) changes in the levels or removal of BDNF or its tyrosine kinase receptor TrkB depletes the pro-survival signals that these molecules provide and inhibits striatal cellular functionality [216] and d) reductions in endocannabinoids and mutant Htt mediated disruption of their transcription which could in turn influence neurotransmitter release, synapse plasticity and the ability of striatal cells to respond to excitotoxicity challenges [217-219].

1.3.5 Autophagy

Increasing evidence points to the fact that the wild type Htt has a vital and active role in autophagy and is involved in autophagosome transport and biogenesis processes [220,221]. For this reason, several important clinical autophagy modulation clinical trials are under development that aim to enhance the ability of selective or general autophagic pathways to increase substrate recruitment, lysosomal activity and clear toxic proteins [222,223]. Autophagy is a cellular, protein clearance system that is divided into three main forms microautophagy, chaperone mediated autophagy and macroautophagy [224]. This classification serves to describe the way the cargo is transported to the lysosomes, and degraded by hydrolases. In macroautophagy the mechanism of toxic protein degradation involves autophagosome vesicle formation, its maturation and finally its fusion with the lysosomes [225,226]. In HD, it has been reported that the macroautophagic vacuoles are inefficient in engulfing their cytosolic loads and this in turn leads

to a reduction in their turnover, an increase in their total number in HD cells and can also be linked to the increased levels of protein aggregates and stored lipids [227]. Additionally, an important regulator of the macroautophagic process, the serine/threonine kinase mammalian target of rapamycin (mTOR) has also been reported to be inhibited and this inhibition has been attributed to the presence of the mutant Htt protein [228]. A separate study has also shown that the accumulation of mutant Htt can also stimulate the endosomal-lysosomal system and this in turn can lead to autophagic cell death [229]. Studies also support the notion that the expanded protein loses its ability to reversibly bind to the endoplasmic reticulum, the Golgi complex, maintain the wild type protein cellular localization properties and regulate stress induced autophagy [230-232] all of which have been associated with neuronal dysfunction and neuronal cell death in HD [233]. In chaperone mediate autophagy (CMA) the substrates are selectively chosen for destruction and delivered to the lysosomal membrane [234]. This is in contrast to the macroautophagy process where whole areas of the cytosol are engulfed and targeted to the lysosomes. In HD models, it has been shown that there is a compensatory regulatory mechanism where the CMA autophagic process activity is increased in response to a compromised macroautophagic pathway [235]. This increased activity is mediated through the upregulation of lysosome-associated protein type 2 a (LAMP-2A) and lysosomal HSC70 both of which proteins have been shown to be directly involved in the clearance of Htt [236]. However, with time the CMA autophagic pathway also gradually declines in its ability to compensate for the dysfunction of the macroautophagic system and as a result there is a generalized failure of autophagic clearance. In microautophagy the lysosomal membrane envaginates the material destined for destruction and internalized by the lysosomal lumen, however its molecular components are still not well understood in mammals [237,238].

1.4 Huntington's disease therapeutic strategies

1.4.1 Transcriptional activity restoration strategies

One of the potential methods to correct for the transcriptional deregulation observed in HD is through the inhibition of histone deacetylase activity. Histone deacetylase inhib-

itors such phenylbutyrate and sodium butyrate have been previously used in an attempt to improve transcriptional dysfunction by the relaxation of DNA conformation, target HD specific pathological events such as caspase mediated cell death [239,240] or even improve HD phenotype through common neurological mechanisms such as improving neuronal BDNF trafficking [241]. In studies where histone deacetylase inhibition was used in HD models, it improved motor performance, appearance and body weight of the mouse models. It has been shown that deacetylase inhibition also reverses mutant huntingtin incited expression and causes a potentially beneficial change in the expression of a specific set of genes associated with the immune system and cell cycle – death, in the cerebellum, the cortex and the striatum of HD mice [242]. Apart from the inhibition of genome-wide transcriptional regulators such as histone deacetylate inhibitors, the transcriptional regulation of individual genes important for the HD has also been suggested as an alternative approach. Examples of such genes are the cAMP response element-binding (*CREB*) and CREB co-activator CBP, brain-derived neurotrophic factor, *REST* as well as the zinc finger transcription factor *SP1*. CREB signaling function has been shown to be reduced in HD and several studies have attempted to increase CREB levels in an effort to improve neuronal survival [243]. Giampa et al. showed that in a rat HD model phosphodiesterase type 10 inhibition increased CREB levels in spiny neurons and provided a neuroprotective effect [244]. Finally, in order to control pathological transcriptional deregulation, several compounds that affect transcription have been used such as anthracycline antibiotics [245]. Stack et al. showed that anthracycline administration in R6/2 HD mice improved nucleosome dynamics and the disease neurological phenotype, shifting the balance between histone methylation and acetylation, while Ryu et al. demonstrated that mithracyclin suppressed *SP1, SP3* related transcription in neurons through the suppression of the ESET (H3-K9) histone methyltransferase and increased HD mouse model survival rates [246,247].

1.4.2 Mutant huntingtin lowering strategies

Silencing of mutant huntingtin by RNA interference notably using siRNAs directed towards the mutant mRNA has become one of the most promising HD therapeutic trials [248]. By blocking the expression of the mutant protein the gain of function effects of

the mutant Htt can be potentially reversed [249]. However, in this strategy it is important to prevent the silencing of the wild type protein as well as other non–specific silencing events [250,251]. In this way, the beneficial effects of silencing can be maximized and patient safety is ensured by avoiding toxic side-effects of non-specific silencing, notably, of wild type Htt that has been associated with perinatal lethality and motor and cognitive defects in specific HD mouse models [252,253]. Even though the first attempts at silencing the mutant protein were performed using RNA interference (RNAi) technologies [254] subsequent studies showed that delivery and efficacy could be improved through the use of single stranded small inhibitory RNAs (siRNAs) and by using chemical modifications and design improvements of the administered molecules [255]. Moreover, additional experiments that have also shown great promise have been targeting the silencing of the mutant protein through the use of antisense oligonucleotides (AONs). The main difference between AONs and RNAi is that while AONs are delivered as single strand molecules RNAis are delivered as delivered as duplexes. Furthermore, RNAis are consequently engaged by the Argonaute protein family as the catalytic components of the RNA-induced silencing complex (RISC) lead only one of the two strands to eventually bind the complementary RNA [256]. Even though RNAi and AONs recognize their targets through Watson and Crick base pairing rules additional key differences that separate the two technologies are that compared to AONs RNAi works through a well-defined RNA degradative pathway (Drosha/Dicer), their targeting efficiency towards the mutant protein, the delivery efficiency to the targeted tissue and their half-life within the administered tissue cells [257,258]. Furthermore, since the silencing of wild type huntingtin function is undesirable due to its vital function in a plethora of cellular, neurological and neurodevelopmental processes [259] a lot of effort has been put into the allele selective silencing of the mutant protein, something that can be done through the targeting of single nucleotide polymorphisms [260,261]. Since the first silencing studies in which a significant reduction of mHtt was achieved through the administration of siRNA and anti-Htt constructs [262,263] a lot of preclinical HD model, Htt lowering studies have already been performed [264]. Some of the most recent, promising studies include those that incorporate the administration of cholesterol-conjugated Htt-siRNA in mice in order to increase tissue uptake [265], the allele specific knockdown of mHtt with S-constrained-ethyl (cET) motif AONs [266] and the AONs RNase-H-mediated

degradation of huntingtin through administration in HD mice cerebrospinal fluid [267].

1.4.3 Mutant huntingtin clearance and anti-aggregation strategies

In order to address the issue of the expanded mutant huntingtin resulting to a toxic gain of function a potential therapeutic strategy has been to identify modulators that possess anti-aggregational roles and that can lead to mutant protein aggregation and inclusion body reduction. A good example of experiments that attempt to reduce the accumulation of aggregated mutant proteins is through the use of chaperone protein member families. Chaperone proteins such as HSP40 and HSP70 have been known to promote refolding and degradation of misfolded proteins [268,269]. In a similar approach Labbadia and colleagues showed that induction of the heat shock response (HSR) via the heat shock factor 1 transcription factor (HSF1) and inhibition of chaperone HSP90 improved HD related phenotypes in the R6/2 mouse model [270]. A subsequent study from the same group and in HD transgenic mouse crossbreeds overexpressing the neuronal chaperone HSJ1a (DNAJB2a) reduced mutant huntingtin aggregation and enhanced solubility [271]. Nonetheless, some of the above ameliorative effects, of the activation and overexpression of the chaperone machinery, have been transient and it has been suggested that synergistic combination therapies might be required for these therapeutic strategies to be more effective [272].

Other anti-aggregation approaches include those that use the administration of various, nontoxic, soluble disaccharides that act to stabilize the partially unfolded mutant huntingtin and prevent disease inclusion formation. One such compound, trehalose has been used in HD as well as other polyglutamine and neurodegenerative disease models and has shown to prevent neurodegeneration, enhance autophagy and alleviate disease mediated pathology [273-275]. Finally, much evidence suggests that cellular huntingtin clearance through induction of autophagy can also provide therapeutic benefits [276-279]. Apart from potential small molecule regulators of autophagy like trehalose and as described above, other approaches to regulate autophagic degradation of mutant huntingtin include: a) post-translational modifications of the mutant protein such phos-

phorylation of specific N-terminal serine residues [280,281] or acetylation of lysine 444 (K444) [282] b) autophagy induction through mTOR inhibition with rapamycin [283] or other mTOR dependent pathways and molecules [284,285] c) through other mTOR independent autophagy inducers such as L-type Ca2+ channel antagonists, K+ATP channel openers and other pharmacophores [286-288] .

1.4.4 Mitochondrial and synaptic function restoration strategies

Since mutant huntingtin has been shown to interfere with mitochondrial energy production and metabolism [289,290] it has been hypothesized that the administration of compounds and molecules that could replenish the energy cellular banks and improve the energy deficient profile in patients may also represent a likely therapeutic pathway. One of the molecules that has been proposed to have the potential to rectify the energy deficit in HD patients is creatine [291]. Creatine is synthesized in the kidney and the liver, taken up by tissues such as brain, heart and the skeletal muscle through a sodium dependent transporter [292] and together with phosphocreatine serve as short term energy banks when energetic demands rapidly increase [293]. Creatine has been shown to improve motor performance and reduce brain atrophy as well reverse mitochondrial dysfunction in transgenic mouse models of HD [294,295]. Additionally, a 16-week clinical trial of creatine administration showed that creatine was well tolerated and reduced oxidative stress levels in HD patients [296]. However even though creatine was also shown in a subsequent study [297] to improve cortical thinning, other aspects of the disease such as cognition were not affected. Therefore, considering the potential side effects of long term creatine administration, especially in presymptomatic individuals, further studies will be required to prove the applicability of this compound in HD. Another corresponding molecule, with neuroprotective and antioxidant properties, is the electron transport chain cofactor coenzyme q10 (CoQ10) which is involved in aerobic respiration and ATP energy production. CoQ10 has in some cases been combined with creatine administration to provide additional neuroprotection in HD as well as Parkinson's disease mouse models [298]. Multiple HD transgenic mouse models have demonstrated the neuroprotective properties of CoQ10 and shown that it can protect dopaminergic neurons, reduce

mitochondrial toxins as well as improve mouse model rotarod performance in combinatorial, preclinical trials [299,300]. It should be noted however that the results of clinical studies of coenzyme Q10 [301] have not been so promising as HD mouse model studies and considering the low treatment benefit compared to patient effort and energy requirement, further clinical trials of this molecule have been currently halted.

Along with mitochondrial restoration strategies much attention has been given to the restoration of cortico-striatal synaptic function in an effort to prevent selective vulnerability and neurodegeneration. The restoration of synaptic function has been the aim of many preclinical and clinical trials to regulate the release of neurotransmitters such as dopamine and glutamate, the functioning of neuroreceptors such as the N-methyl-D-aspartate receptor (NMDAR) and the expression levels and the release of neuromodulators and growth factors such as the endocannabinoids and brain derived neurotrophic factor [302]. In particular, experiments on transgenic R6/2 mouse models showed that dampening of the aberrant glutamate uptake by activation of metabotropic glutamate auto-receptors, resulted in increased model survival and a significant rescue in striatal and cortical neuronal loss [303]. Another attempt to prevent striatal loss and atrophy in HD as well as other neurodegenerative diseases has been through the overexpression of BDNF [304]. In HD, it has been shown that the increase of BDNF levels in YAC128 mouse models has resulted in the normalization of dopamine D2 receptor expression while striatal cell loss and motor dysfunction have also been reduced [305]. Finally, additional synaptic rescue schemes include the modulation of synaptic transmission via endocannabinoid signaling [306,307] and administration of neurotransmission inhibitory drugs as riluzole, memantine and the dopamine pathway inhibitor tetrabenzine [308-311].

1.4.5 Other strategies

Cell replacement therapy is another favorable approach that can potentially delay disease progression by replenishing the cells that have been lost due to the pathological effects of the mutant Htt protein. To date, cell therapy approaches have been through the use of stem cells, neural progenitor cells as well as fetal tissue cells [312]. El-Akabawy et al.

showed that the implantation of human neural stem cells in the R6/2 mouse model had suboptimal results due to low cell survival and differentiation. However, a different study by Rossignol et al. showed that co-transplantation of adult mesenchymal and neural stem cells in a transgenic rat model prolongs the survival of the neural stem cells and creates a more favorable transplantation microenvironment [313,314]. Transplantation of fetal striatal grafting has also been shown to improve the motor as well as the cognitive decline of HD patients. Nonetheless the grafts exhibited poor survival over time and conversely the grafting beneficial effects diminishing over time [315,316]. The poor survival of the grafts in the host has been attributed to poor vascularization. Trophic support can potentially be improved as grafting techniques and protocols are continuously improved [317,318]. Nevertheless, achieving the delicate balance of the type of stem cells to use (MSCs or iPSCs), during which HD disease stage (early, middle or late) and the specific neurodegeneration events these cells will target, are the greatest challenges of future stem cell trials and the determinant success factor for this therapeutic method [152].

To conclude the list of therapeutic approaches it has been suggested that targeting caspase dependent cleavage events is another prospective approach for the prevention of neuropathological features in HD and other neurodegenerative disorders [319]. According to the "toxic fragment hypothesis" the presence of multiple proteolytic sites on the Htt protein, in combination with the aberrant activity of proteases such as caspases and calpains may lead to numerous, variable length polyglutamine containing peptides that can be toxic. The identification of the specific combinations of enzymes and sites causing the most toxic peptides has been the focus of several research groups. Using YAC HD mouse models it has been shown that mice expressing mutant huntingtin resistant to caspase 6 cleavage, were protected against neurotoxic events and did not develop striatal neurodegeneration [320]. For this purpose, drugs like minocycline have been used in preclinical trials and in an attempt to inhibit the production of huntingtin toxic fragments by caspase cleavage [321,322]. Furthermore, initial clinical trials of minocycline use have exhibited stabilization of motor function and a significant amelioration of psychiatric symptoms in HD patients [323]. Even though the role of caspases is still not completely understood and the experimental results are not always consistent with the initial results from the caspase 6 resistant YAC mice [324], new molecules are constantly being

developed against caspase-6 aiming to increase the therapeutic potential of this method [325,326]. Yet increasing evidence points at important non-apoptotic roles of caspases such as caspase-6. Furthermore, alternative, complementary proteolytic pathways exist, which continue to give rise to the HD proteolytic toxic peptides (even after ablation or downregulation of caspase-6 function). This means that research in this direction should proceed with caution as to the molecules used and the extent to which the function of these proteases can and should be modified.

1.5 Biomarkers

1.5.1 What is a biomarker?

A biomarker is a physiological, molecular, biochemical or pharmacological measurement that can be obtained from the body of a patient and that can provide a good indication of disease prediction/progression or treatment efficacy in that patient [327]. Additionally, and according to the US Food and Drug administration (FDA) pharmacogenomics guidance (http://www.fda.gov/drugs) a biomarker should be measured in an analytical test system with clearly established performance characteristics and for which there is an established scientific framework that elucidates the significance of the biomarker test results. A good biomarker should also have the following properties: a) be able to detect disease features with high sensitivity (able to detect the condition when it is present) and high specificity (able to rule out a condition when it is absent), especially when compared to diseases with similar phenotypes and b) be easily, reliably and robustly detected, preferably in an easily collectable tissue (such as peripheral blood) and with minimum distress to the patient [328]. Finally, a good biomarker is judged by its positive predictive power (proportion of patients with a correct positive diagnosis over the total number of individuals who have been called to have the disease, true and false) and its negative predictive value (proportion of patients with a negative test and a correct diagnosis over the total number of people that have been called to not have the disease, including the false negatives).

Based on their disease specific properties and their relationship to disease, pathology

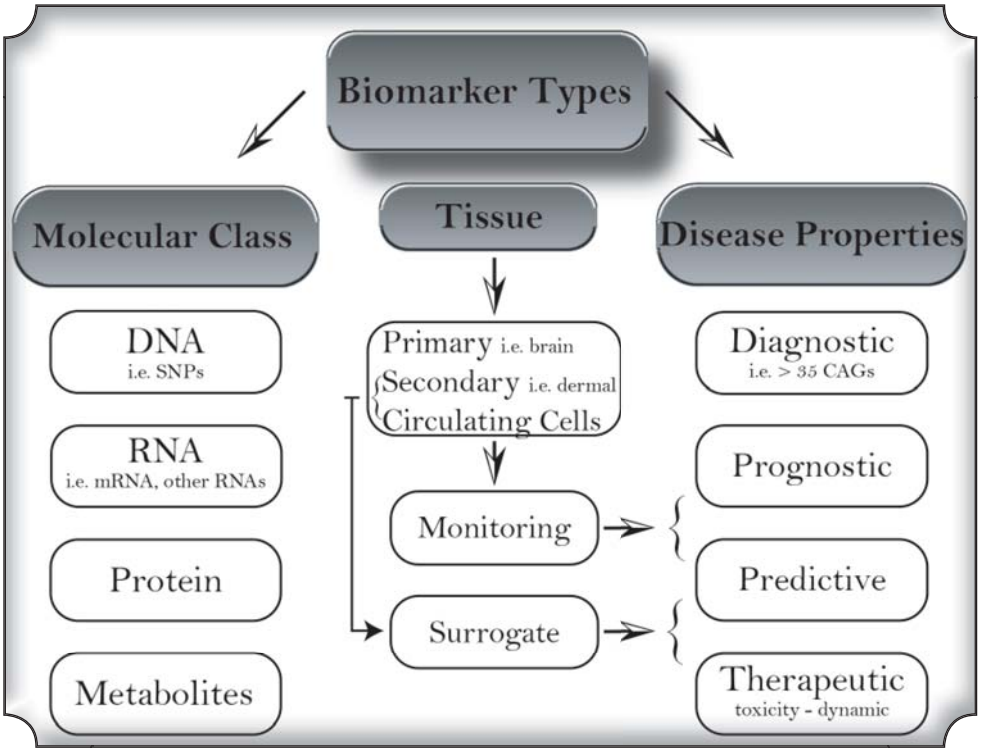


Figure.1 Biomarker type classification based on: a) the molecular subclass from which the identified biomarker originates b) biomarker tissue relative to disease central pathology tissue and c) biomarker provided information relative to disease risk (diagnostic), disease progression (prognostic), therapeutic potential (predictive) and safety or effectiveness of therapeutic approach.

biomarkers can be classified into: a) biomarkers which predict patient survival (prognostic), b) biomarkers which follow disease progression (monitoring biomarkers), c) biomarkers which can be used to evaluate drug safety and efficacy (pharmacodynamic), d) biomarkers which identify individuals likely to benefit from a treatment (predictive) and e) biomarkers which can predict the outcome given the response to therapy (surrogate biomarkers– see Figure 1) [329]. Prognostic and diagnostic biomarkers are grouped together and are otherwise called type 0 category biomarkers while therapeutic and surrogate biomarkers have been defined as type 1 and type 2 biomarkers respectively [330]. A prognostic biomarker can provide information about the outcome of the disease in

specific population groups however it cannot provide information about the patient's response to a treatment such as those provided by predictive biomarkers. On the other hand, pharmacodynamic markers can provide information about how well the treatment works (proof of principle) or can be used to optimize the dose of the administered treatment. Surrogate biomarkers are measurements that can potentially replace clinical endpoints and this is performed in a less labor intensive or expensive way than the standard and established clinical endpoint measurements methods for the specific disease. A biomarker can be an intrinsic measurement such as those provided by brain or other tissue imaging techniques, biomolecular and fluid sample tests or they can also be extrinsic. For example, an extrinsic surrogate biomarker can be cigarette smoking to the clinical end point of lung cancer. Moreover, biomarkers can be divided to the level to which they take place in a disease (molecule, cell etc.) or even according to their relationship to disease pathology (primary or secondary pathology) [331]. A good biomarker should also undergo validation and qualification [332]. Validation refers to the evaluation of the biomarker's sensitivity, specificity and reproducibility while on the other hand biomarker qualification refers to acquisition of the evidence linking a biomarker to a specific clinical endpoint [333]. Even though a biomarker can be linked, validated and qualified to a clinical endpoint (surrogate biomarker) this may not always be associated with disease causality [334]. Specifically, such a biomarker may be associated with the clinical endpoint but could nonetheless have nothing to do with the disease tissue pathology or the molecular pathways that lead to the disease specific endpoint. Such a biomarker could for example be a measurement in the peripheral blood of neurodegenerative disease patients not correlated to brain degeneration and its associated molecular pathways and yet correlated to specific disease endpoints. Finally, it should be emphasized that apart from the disease specific, drug and disease endpoint biomarker relationships, vital properties of a biomarker should be the detailed reporting of laboratory, experimental, clinical and statistical information that will allow for the unambiguous replication and reconstitution of the initial biomarker study results [330,335].

1.5.2 Biomarkers in Huntington's disease - needs and challenges

Different biomarkers can be developed for different clinical purposes. No diagnostic bio-

markers are needed for HD as diagnosis is done by the genetic identification of the number of CAG repeats. Thus, HD biomarker development is focusing on the areas of pharmacodynamics, therapeutic monitoring and disease progression diagnosis. Additionally, the ability to identify potential HD mutation carriers provides a period of several years to decades that can be used for presymptomatic therapeutic intervention. This is further emphasized by the fact that even though individuals exhibit no distinct phenotypic features during this preclinical, presymptomatic disease stage, subtle subclinical molecular and neurodegenerative events are already taking place [336-339]. Another reason that identification of good biomarkers is a top priority for HD research is the recent development of many potential treatments and therapeutic molecules (see 1.4). These therapeutic strategies have been partially validated with the help of various HD models. However, unless effective and standardized biomarkers have been described it will be very difficult to objectively describe any ameliorative outcomes of potential treatments when patients progress to the clinical trial stages. Such biomarkers should ideally describe the disease progression or even the pathology reversal equally well in early and presymptomatic patients, considering that these patients would benefit the most from therapeutic intervention. Nevertheless, even though high standards are required for the discovery of sensitive biomarkers, the multiplicity of pathological features in HD dictate that more than one type of biomarkers will be required to accurately describe the disease progression and the clinical trial benefits. Such a panel of biomarkers would be taking advantage of clinical progression tests, technological developments in imaging technologies and refinement of biochemical markers already under development. For Huntington's disease as well as other neuropsychiatric disorders, one of the main problems in studying the actual disease pathology is that it is hardly possible to collect tissue samples from brain areas of interest or obtain representative patient sample numbers to achieve the desired statistical power for a conclusive result [340,341]. Additionally, postmortem samples, which are sometimes also used to study the disease, are usually of suboptimal quality, either due to postmortem delay or due to pathological events affecting the tissue for several years, like in Huntington's disease [342-344]. Finally, development of qualified (see 1.5.1) and informative pharmacodynamic, monitoring and surrogate biomarkers will not only aid in the development of clinical trials and therapeutic agents but also assist researchers to get a better understanding of the disease pathology itself.

1.5.3 Blood based biomarkers for Huntington's disease

As described above HD patients are often irritable and exhibit choreatic symptoms and psychiatric disturbances (see 1.1.3/1.1.4). This makes it even more challenging to perform biopsies and secondary pathology tissue samplings and examinations in symptomatic patients. At the same time the ease with which peripheral blood can be collected repeatedly over a period of time, makes it a very attractive tissue for use in preclinical, clinical and biomarker discovery studies. Furthermore, huntingtin is also expressed in blood and, as such, surrogate biomarker changes could be identified in blood independently of potential causality to central disease pathology. The changes found in blood could reflect the effect of the mutant huntingtin itself within the tissue or secondary pathological events from other tissues, such as muscle. For example, it has been suggested that in neurodegenerative diseases the blood-brain barrier might be compromised, potentially exacerbating of brain pathology and causing leakage of proteins or other molecules [345]. Finally, profiling of gene expression in HD patients has shown that in whole blood and caudate samples transcription of large genomic regions is regulated in a synchronized fashion [346]. However, independently of the pathological mechanisms or other events traced by blood biomarkers they will need to be robustly associated with well-established HD clinical endpoints.

Blood consists mainly of plasma (55%), red blood cells (45%) and leukocytes and platelets (last two together ~ 1%). Most blood HD studies have been performed either using plasma and serum to examine the free proteins and molecules in these liquids; or using the leukocytes to examine cellular organization and disease related processes. The blood component or molecule under examination is often chosen based on the suspected deregulated disease pathway. By following one such hypothesis-driven approach and to study oxidative damage and dysfunction of the mitochondria in HD, serum levels of 8-hydroxy-2'-deoxyguanosine (8OH2'dG), an oxidative DNA damage indicator, have been investigated [296,347,348]. Its levels have been reported to be elevated in HD and other neurodegenerative diseases and HD mouse models overexpressing a hydrolase that break it down have conferred a protection against HD-like symptoms [349,350]. Additionally, its pharmacodynamic potential was tested by comparing its levels in HD

individuals before and after CoQ10 treatment [347] (for role of Coq10 in HD see 1.4.4). Nevertheless, the results have not always been consistent with the initial positive findings and it has been proposed that 8-hydroxy-2'-deoxyguanosine has only limited potential at assessing clinical disease features [351-353]. A more direct approach of disease specific biomarker identification involves the measurement of the mutant huntingtin itself in circulating T cells, B cells and monocytes [354,355]. The results of the study performed by Weiss et al. showed that mHtt leucocyte levels were lower in presymptomatic patients when compared to symptomatic and were also associated with disease burden and caudate atrophy scores. The quantification of mHtt itself as a biomarker obtained from peripheral readings such as blood or other peripheral fluids such as cerebrospinal fluid (CSF) can be very important since it can identify the effect of mutant protein lowering therapeutic strategies (pharmacodynamic) (see 1.4.2) ; can be indicative or associated with peripheral inflammation and immune system deregulations (see 1.2.4) (monitoring – predictive biomarker properties); and it can provide mechanistic links and clues to disease pathology. For example, the latter can be achieved by measuring mutant protein relationships in blood and other peripheral tissues, such as the relationship of soluble and aggregated mutant huntingtin levels [356]. Since it is not yet clear to which extent the interplay between aggregated and soluble mutant proteins forms influences neuronal cellular toxicity, the delineation of the relative amounts of the above two species or conformers can provide additional clues to the disease pathology and constitute on its own a type of biomarker. Other individual molecule and compound findings that have been described in HD blood include increased cytokine levels (mainly IL-6, IL-8 and TNF-a), kynurenine : tryptophan ratios as markers of microglial activation and persistent inflammation [357,358] and lower levels of BDNF, which is associated with striatal neuron survival signals, in HD patients serum and it association with longer CAG repeats [359].

Apart from specific hypothesis driven approaches, as illustrated above, peripheral blood has also been used in non-hypothesis driven efforts to identify peripheral biomarkers. Such non-hypothesis driven methodologies take advantage of state of the art high-throughput techniques such as mass spectrometry and genome wide gene expression profiling platforms to identify additional peripheral molecular biomarkers, among others metabolites and mRNAs [360,361]. To identify changes in mRNA expression levels in HD scientists

have mainly used whole peripheral blood and isolated leukocyte samples of patients as well as animal models [362-366]. Even though the overlap between studies result has not been very good for the mRNA changes identified, the per study findings have demonstrated that blood holds potential in being used as a source for disease progression and therapeutic efficacy. For example, Hu et.al. have demonstrated that dynamic regulator of chromatin plasticity H2A histone family (*H2AFY*) is overexpressed both in the blood and frontal cortex of HD patients, that this association precedes clinical symptoms and that the levels of this marker respond to a neurodegeneration suppressor, thus implying pharmacodynamic potential [364]. The above biomarker also contains many of the properties of an ideal biomarkers as it is validated in HD mouse models, changing according to disease severity and found early in the disease allowing for a wider therapeutic intervention window. Moreover, it has a known mechanism in disease pathology i.e. chromatin regulation (for the role of chromatin regulation in HD see 1.3.2) [367,368]. Apart from gene expression platforms metabolomics and proteomics technologies have been widely used to also identify such biomarkers in HD blood. Notably metabolomics could be a promising approach as deregulation of metabolism is a known HD pathology. Metabolomics and proteomics studies have also provided much information about the changes in patient blood samples [141,369-371]. For instance, proteomic and metabolomic changes in HD blood include altered levels of brain derived metabolites such as 24S-hydroxycholesterol in pre-manifest carriers, associated with metabolically active neuronal cells [372]; the presence of a catabolic phenotype in early HD patients, associated with aliphatic amino acids and changes in fatty acid breakdown markers like glycerol and malonate [373] and identification of neuroinflammation activation proteins in plasma [138]. Together all of the above results show that blood has great potential to provide prodromal, monitoring as well as pharmacodynamic HD biomarkers.

1.5.4 State of Huntington's disease clinical and imaging biomarkers

The identification of clinical biomarkers involves a series of tests and examinations that have the purpose of measuring cognitive and neuropsychiatric condition and motor function performance of mutation carriers. One of the best known of these tests

and the standard method of assessment of clinical disease progression is the Unified Huntington's Disease Rating Scale (UHDRS) [374]. This score comprises of different sub-scores that assess the patient's motor performance with tests such as oculomotor and finger tapping performance, tongue control and force, grip and posture control but also tests assessing their cognitive and functional condition. The advantages of such clinical measures are that they are usually inexpensive, standardized, relative quick and noninvasive. On the other hand, the disadvantages are that they are subjective and display inter and intra-rater variability [375]. However, despite potential disadvantages of some clinical markers there have been organized efforts such as the TRACK-HD and PREDICT-HD consortia that aim to improve the objectivity of these clinical measures as well as providing higher longitudinal change sensitivity and cross sectional differences in presymptomatic patients [376-380]. For example, 36-month observational data from the TRACK-HD study showed that several quantitative motor and cognitive tasks showed increased rates of decline. Furthermore, psychiatric markers such as apathy also exhibited significant increases [379] while the PREDICT-HD study reported that symptoms such as depression, anxiety and obsessive-compulsiveness were present more in HD mutation carriers [381].

A group of HD biomarkers that have produced some of the most significant results during the last years have been collected with the help of (neuro)imaging technologies. These technologies have the ability to describe brain loss changes as well as brain morphological changes in HD mutation carriers. The advantages of imaging techniques are that they non-invasive and that the data produced are in general well reproducible [382]. On the other hand, imaging techniques are usually quite expensive, especially when compared to clinical scoring systems. An additional disadvantage is that these examinations are sometimes difficult to perform due to the structural design of the imagers and the fact they can cause claustrophobia or are even unsuitable for patients that exhibit highly choreatic movements [376]. One of the most used of these technologies is magnetic resonance imaging. MRI studies have demonstrated how cortical degeneration is related to the clinical symptoms and how the cortical changes develop in different patients. One such prominent study by Rosas et al. showed that in HD subjects thinning of the cortical ribbons takes place from the posterior to anterior regions, that these changes take

place early in the disease and could be accountable for the disease heterogeneity [383]. In a subsequent study these patterns of cortical thinning were associated with different motor phenotypes, while functional and cognitive scores were closely related to specific cortical areas [384]. Other promising imaging techniques include functional MRI, PET, diffusor tensor imaging and magnetic resonance spectroscopy. These techniques can offer information about brain area dynamic changes, functional interconnectivity and microstructural degeneration [385,386]. Notably data from the IMAGE-HD study, using quantitative susceptibility mapping and multimodal magnetic resonance imaging, has shown that presymptomatic patient brain areas exhibited a higher iron content and that caudate volume loss could separate presymptomatic HD mutation carriers from controls respectively [387,388]. When it becomes possible to combine complementary information from different imaging types in longitudinal studies, this will greatly improve the potential and the sensitivity of imaging as a biomarker source. However, such an effort is not a small task and it will require greater standardization as well as the integration of different classes of professionals such as doctors, clinical researchers and statisticians [389].

1.5.5 Other molecular and biochemical fluid biomarkers

Aside from blood and the relative advantages it offers compared to other tissues, cerebrospinal fluid (CSF), muscle, saliva and urine have also provided useful and informative "wet" biomarkers for HD. The use of CSF is becoming increasingly popular in monitoring disease progression in other major neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [369,390-392]. CSF advantages include the fact that it is also relatively accessible and that it interacts with central nervous system tissues and is "open" to brain derived proteins which can potentially mirror the pathological changes in the brain tissue. Wild et al. were able to reliably quantify mHtt at femtomolar sensitivity in mutation carriers' CSF and also showed that higher mHtt load was correlated to reduced cognitive function and increased motor dysfunction in HD patients [393]. In a separate study, Tan et al. used postmortem samples to show that CSF possesses an mHtt and tissue specific property called cell-based aggregation (CBA) seeding, that seeds mutant huntingtin aggregation. This provides a mechanistic explanation for disease pa-

thology and can also act as a potential molecular biomarker for HD [394]. This CSF aggregate seeding propensity could be used to track disease progression (i.e. constituting a monitoring biomarker) by measuring CBA activity in a cell based assay. Saliva is another tissue that has been used to identify disease biomarkers, notably to study neuroendocrine regulation disturbances such as the hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and depression found in HD carriers. Cortisol concentrations obtained from HD mutation carriers' saliva samples were increased, indicative of HPA axis dysfunction and also exhibited a correlation with motor and functional scores [395]. It should be noted however that the association of HPA axis activity with HD disease stages (pre-symptomatic vs (motor)symptomatic) and psychiatric symptoms (depression, suicidality) is not yet clear and further longitudinal studies are required for such saliva measurements to constitute potential biomarkers [396,397]. Additional examples of the use of other accessible fluids and tissues in biomarker identification include the reports of progressively increased cortisol levels in patient urine samples [398], the reduction of muscle contractility markers and a switch from fast to slow twitch muscle types in skeletal muscle of patients [399]. Finally, the function of the autonomic nervous system (ANS) in Huntington's disease has also been investigated to explore how this is affected in patients [400]. When standardized ANS system tests (sympathetic skin responses, blood pressure, heart rate variability etc.) were performed in patients, autonomic symptoms were found to be related to HD functional disability and depression and autonomic deregulation correlated with central nervous system degeneration tests and scores, such as the symbol digit modalities test and the UHDRS motor score [401,402]. These results support the notion that autonomic functional assessment could present a complementary non-invasive measure of disease progression.

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