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Increased levels of hemoglobin and α_1-microglobulin in Huntington's disease

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1. ABSTRACT

Hemoglobin released from damaged erythrocytes is a major pro-oxidant, generator of free radicals and inflammatory mediator. Huntington's disease is an inherited neurodegenerative disorder characterized by both neurological and systemic abnormalities, in which oxidative stress has been suggested as a possible pathogenic mechanism. In the present work we have investigated levels of hemoglobin and markers of oxidative damage, including the heme- and radical-scavenger α_1-microglobulin, in plasma and urine samples from two separate sample cohorts, including controls, premanifest gene carriers and subjects at different stages of Huntington's disease. The results show statistically significant increased levels of hemoglobin and α_1-microglobulin in Huntington's disease urine samples. Interestingly, urine hemoglobin levels correlate with clinical severity. The results suggest that hemolysis may be linked to the pathogenesis of Huntington's disease and that assay of hemoglobin and α_1-microglobulin may provide biomarkers that are linked to biologically relevant processes.

2. INTRODUCTION

Huntington's disease (HD) is an inherited neurodegenerative disease, caused by a CAG triplet repeat expansion in the gene encoding huntingtin, for which there is no effective disease-slowing treatment. Many clinical features of HD can be ascribed to neuronal dysfunction and death but evidence is emerging suggesting a role for extra-neuronal cells and tissues in the pathogenesis of HD. In addition to the classical symptoms of chorea, cognitive decline and psychiatric symptoms, HD manifests with peripheral features, such as weight loss and skeletal muscle wasting (1) and the causative mutant huntingtin protein is expressed ubiquitously (2).

Mitochondrial abnormalities and oxidative damage have been suggested as important features of neurodegenerative brain pathology. In HD, there is evidence from both *in vivo* and *in vitro* studies showing oxidative damage to DNA, lipids and proteins (3). Furthermore, markers of oxidative damage have been found upregulated in other neurodegenerative conditions, including Alzheimer's disease (4).

Table 1. Demographic characteristics of subject cohorts

	Disease stage	Number of subjects	Female:male	Mean age (range)	Mean CAG (range)
Cohort 1					
Urine samples	Control	65	39:26	42 (21-74)	-
	Premanifest	14	9:5	40 (31-61)	-
	Early	26	15:11	45 (23-79)	-
	Moderate	25	16:9	49 (22-70)	-
Plasma samples	Control	65	39:26	42 (21-74)	-
	Premanifest	14	9:5	40 (31-61)	-
	Early	26	15:11	45 (23-79)	-
	Moderate	25	16:9	49 (22-70)	-
Cohort 2					
Urine samples	Control	20	10:10	43 (29-58)	-
	Premanifest	20	12:8	42 (29-54)	43 (40-47)
	Early	20	10:10	47 (25-63)	44 (41-52)
	Moderate	13	8:5	51 (36-75)	45 (42-50)
Plasma samples	Control	19	9:10	41 (29-53)	-
	Premanifest	18	10:8	42 (29-54)	43 (40-47)
	Early	19	10:9	46 (25-63)	44 (41-52)
	Moderate	11	7:4	52 (38-75)	44 (42-49)

Oxidative damage is caused by reactions between cell- and matrix biomolecules and oxidants such as reactive oxygen species (ROS), including the free radicals superoxide and hydroxyl radical. Hemoglobin (Hb), one of the most abundant proteins in blood, is normally contained within red blood cells but is a major generator of ROS when released in free form in plasma and at extravascular sites (5). In addition, its downstream metabolites heme and free iron are also potent inducers of oxidative damage (6). Thus, Hb-induced oxidative damage often leads to cell- and tissue damage.

α_1-microglobulin (A1M), a 26-kDa tissue and plasma glycoprotein which is synthesized in liver and secreted to blood (7), is a radical- and heme-scavenger and has heme-degrading properties (8, 9). Antioxidative protection effects of the protein have been shown in cell culture models where oxidative damage was induced by free Hb, heme and ROS (10). It is suggested that A1M is involved in the physiological defence against Hb-induced oxidative stress since the expression of the protein is upregulated *in vitro* and *in vivo* by Hb, heme and ROS (11, 12).

Reliably quantified urine and/or plasma markers that track with disease progression would be an asset in future clinical trials in HD. Therefore we aimed to investigate whether an altered composition of oxidative markers is associated with progression of HD. More specifically, we evaluated levels of Hb, A1M, protein carbonyl groups and the lipid peroxidation product malonyldialdehyde in urine and plasma from HD patients and control subjects.

3. MATERIALS AND METHODS

3.1. Collection and processing of human samples

The study was conducted in accordance with the declaration of Helsinki and was approved by local ethics review boards; all subjects gave informed written consent. Blood and urine samples were obtained from control subjects, genetically-diagnosed HD premanifest subjects and HD patients at different disease stages, i.e. early and moderate. To insure limited effect of sampling time, all samples were collected between 2 and 4 pm, from non-fasting subjects at the National Hospital for Neurology and Neurosurgery Huntington's Disease Multidisciplinary Clinic using standardized biomarker sampling protocols and blood samples were processed as previously described (13). Clinical assessment was carried out by a neurologist experienced in assessment of HD patients. Total functional capacity (TFC) scores were used to define early vs. moderate HD. Early HD encompasses stage 1 and 2 and is defined by a TFC of 7-13 and moderate HD is defined as a TFC of 3-6 (14). Demographic and clinical data of patients and controls are given in Table 1.

3.2. Reagents and proteins

Oxyhemoglobin, used as a standard in the ELISA, was purified as previously described (15) from whole blood, freshly drawn from healthy subjects. Hb, used for coating in the ELISA, 2,4-dinitrophenylhydrazine (DNP-hydrazine), 2-thiobarbituric acid, p-nitrophenyl phosphate and O-phenylenediamine were purchased from Sigma-Aldrich (Stockholm, Sweden). Human A1M was purified from urine as described (16). Goat anti-human A1M was prepared as previously described (17). Rabbit anti-human Hb (IgG purified) and alkaline phosphatase-conjugated swine anti-rabbit IgG were purchased from Dako A/S (Glostrup, Denmark). Anti-DNP-keyhole limpet hemocyanin (KLH) was from Invitrogen (Eugene, Oregon, USA).

3.3. Determination of protein carbonyl groups

Formation of protein carbonyl groups, a marker of oxidation events (18) in urine and plasma, were determined as described (19), in blinded duplicates. Briefly, samples were mixed with DNP-hydrazine for 45 minutes in room temperature (RT). The DNP-hydrazine derivatized samples were then diluted in PBS and coated on a 96-well microtiter plate for 2 hours in RT. After rinsing, the plate was incubated with rabbit anti-DNP-KLH (Diluted 1:2000 in PBS, 0.1 % BSA, 0.25 % Tween 20; incubation buffer) for 2 hours in RT, followed by rinsing and incubation with horse radish peroxidase-conjugated swine anti-rabbit IgG (Diluted 1:2000 in incubation buffer) for 1 hour in RT. Finally, the plate was incubated with substrate solution (1

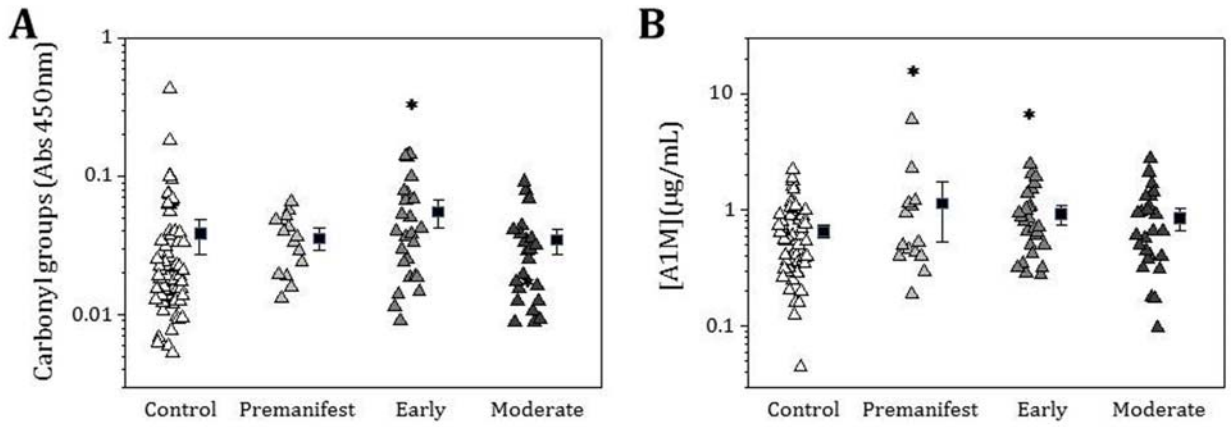

Figure 1. Carbonyl groups and A1M in urine of cohort 1. Samples were from control subjects and genetically-diagnosed HD patients. (A) The levels of protein carbonyl groups were measured in urine using an ELISA method as described in materials and methods. (B) The A1M concentration in urine was determined by RIA as described in materials and methods. The results from the analysis are plotted as a scatter of individual sample data and as mean ± SEM, * P<0.05.

tablet O-phenylenediamine dissolved in 60 mM Tris-HCl, pH 8.5, 50 mM Na_2HPO_4 and hydrogen peroxide) and absorbance was read at 450 nm at the onset of the reaction until a peak absorbance was obtained.

3.4. Radioimmunoassay (RIA)

Radiolabelling of A1M with ^{125}I was done using the chloramine-T method (20). Protein-bound iodine was separated from free iodide by gel-chromatography on a Sephadex G-25 column (PD10, GE Healthcare, Stockholm, Sweden). A specific activity of around 0.1-0.2 MBq/µg protein was obtained. RIA was performed by the polyethylene (PEG)-exclusion method (21). Goat antiserum against human A1M was mixed with ^{125}I-labelled A1M and unknown patient samples or standard A1M-concentrations. After incubating overnight at RT, antibody-bound antigen was precipitated by adding bovine serum and 15 % PEG-6000, centrifuged at 2500 xg for 40 min, after which the ^{125}I-activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Sverige AB, Upplands Väsby, Sweden). Samples were assayed in blinded duplicates.

3.5. Hemoglobin measurement

A competitive ELISA was employed for quantification of Hb (12). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with Hb overnight at RT, and then incubated 2 hours at RT with a mixture of standard Hb or samples, and rabbit anti-Hb. After rinsing, the wells were incubated for 1 hour at RT with alkaline phosphatase-conjugated swine anti-rabbit IgG. Finally, a substrate solution containing p-nitrophenyl phosphate was added, and the absorbance was read at 405 nm at the onset of the reaction until a peak absorbance was obtained. Samples were assayed in blinded triplicates.

3.6. Thiobarbituric acid-assay

The concentration of malonyldialdehyde, a lipid peroxidation product, was measured in urine and plasma by the thiobarbituric acid (TBA) method described by Gutteridge (22). Briefly, urine and plasma samples were incubated in PBS, TBA-reagent (0.5 % 2-thiobarbituric acid and 0.5 % SDS in H_2O) and 0.2 M glycine-HCl, pH 3.6 and the mixture was heated to ~100°C for 15 min. After cooling to RT, samples were centrifuged (8000 xg, 2 min, 4°C) and the absorbance at 532 nm was read). Samples were assayed in blinded duplicates.

3.7. Statistical analysis

Data are presented as mean ± SEM unless otherwise specified. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The significance of differences between groups was evaluated using One-way ANOVA, more specifically, Kruskal–Wallis one-way analysis of variance followed by the Dunn´s multiple comparison test (in the results both the ANOVA p value and the post hoc test p values are indicated). P<0.05 was considered statistically significant. Correlations with clinical variables were examined using linear regression analysis.

4. RESULTS

4.1. Analysis of urine samples in cohort 1

In a cross-sectional sample material (cohort 1, Table 1) we investigated the presence of oxidative modifications in HD by analyzing the levels of protein carbonyl groups in urine of all samples (Figure 1A). Results showed that there was no significant difference in the amount of carbonyl groups when comparing HD (mean value 0.043 ± 0.004) with controls (mean value 0.038 ± 0.007). However, a significant increase was seen in the early group (mean value 0.055 ± 0.009) as compared to the control group (One way ANOVA (Kruskal-Wallis test) p-value 0.03, post hoc Dunn´s test control vs. early p-value 0.02). Also, a slight decrease in the levels of protein carbonyl groups were observed in the premanifest (mean value 0.036 ± 0.004) and moderate groups (mean value 0.034 ± 0.005).

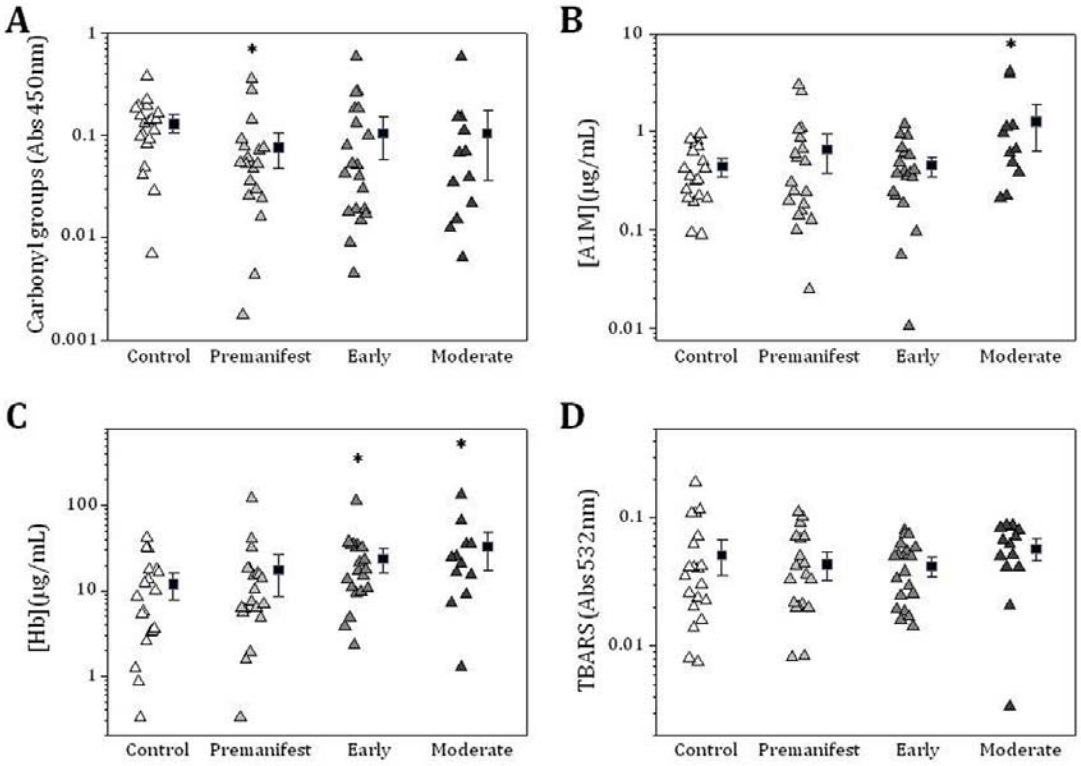

Figure 2. Hb, A1M and markers of oxidation in urine of cohort 2. Samples were from control subjects and genetically-diagnosed HD patients. (A) The levels of protein carbonyl groups were measured in urine using an ELISA method as described in materials and methods. (B) The A1M concentration in urine was determined by RIA as described in materials and methods. (C) The Hb concentration in urine was measured by ELISA as described in materials and methods. (D) The concentration of malonyldialdehyde, a lipid peroxidation product, was measured in urine by the TBA method as described in materials and methods. The results from the analysis are plotted as a scatter of individual sample data and as mean ± SEM. * P<0.05.

The concentration of A1M was measured in the urine samples (Figure 1B) and the concentration was significantly increased in the HD-group (mean value 0.94 ± 0.11 µg/ml, p-value 0.02) as compared to the controls (mean value 0.65 ± 0.06 µg/ml). When comparing at group level, it was seen that concentrations in the premanifest (mean value 1.13 ± 0.40 µg/ml, One way ANOVA (Kruskal-Wallis test) p-value 0.05, post hoc Dunn´s test control vs. premanifest p-value 0.03) and early groups (mean value 0.92 ± 0.12 µg/ml, post hoc Dunn´s test control vs. early p-value 0.02) were significantly increased as compared to controls. Furthermore, the mean concentration of urinary A1M was elevated in the moderate group (mean value 0.85 ± 0.13 µg/ml) compared to controls but not significant (post hoc Dunn´s test control vs. moderate p-value 0.10).

4.2. Analysis of urine samples in cohort 2

To validate our results and investigate more markers in detail we used a separate sample cohort (cohort 2, Table 1). The findings seen in cohort 1 were confirmed in cohort 2, i.e. no significant difference in protein carbonyl group formation could be seen (Figure 2A). Also, the increased concentration of A1M seen in cohort 1 was confirmed in all disease groups in cohort 2 (control, mean value 0.44 ± 0.06 µg/ml; premanifest, mean value 0.67 ± 0.19 µg/ml; early, mean value 0.46 ± 0.07 µg/ml; moderate, mean value 1.27 ± 0.42 µg/ml) and was found to be significantly increased in the moderate group (One way ANOVA (Kruskal-Wallis test) p-value 0.05, post hoc Dunn´s test control vs. moderate p-value 0.02) (Figure 2B).

In light of previous findings, which have shown an association between increased A1M concentration and elevated Hb levels (10-12), we assayed the concentrations of Hb in the samples of cohort 2. The concentrations of Hb in the urine samples (Figure 2C) were significantly increased in early (mean value 24.0 ± 5.4 µg/ml, One way ANOVA (Kruskal-Wallis test) p-value 0.02, post hoc Dunn´s test control vs. early p-value 0.05) and moderate HD (mean value 32.6 ± 10.4 µg/ml, post hoc Dunn´s test control vs. moderate p-value 0.03) compared to controls (mean value 12.0 ± 2.7 µg/ml). The mean concentration of Hb in the premanifest group showed a tendency to be elevated (mean value 17.5 ± 6.0 µg/ml, post hoc Dunn´s test control vs. premanifest p-value 0.41) compared to controls.

Interestingly, Hb levels were found to correlate with disease progression, i.e. Hb levels in urine

Table 2. Biomarkers measured in plasma of cohort 2

	Disease stage	Carbonyl groups	A1M (μg/mL)	Hb (mg/mL)	TBARS
Cohort 2					
Plasma samples	Control (n=18)	0.086±0.013 (0.058 – 0.114)	42.7±2.2 (38.0 – 47.4)	1.20±0.18 (0.81 – 1.59)	0.077±0.006 (0.064 - 0.089)
	Premanifest (n=18)	0.106±0.016 (0.073 – 0.139)	42.0±2.6 (36.5 – 47.6)	1.69±0.37 (0.92 – 2.47)	0.086±0.024 (0.035 - 0.136)
	Early (n=19)	0.089±0.011 (0.065 – 0.113)	45.6±2.9 (39.5 – 51.7)	1.32±0.30 (0.69 – 1.94)	0.074±0.009 (0.055 - 0.092)
	Moderate (n=11)	0.088±0.016 (0.052 – 0.123)	45.9±2.9 (39.4 – 52.5)	1.35±0.26 (0.77 – 1.93)	0.156±0.067 (0.008 – 0.305)

Carbonyl groups, A1M, Hb and TBARS were determined in plasma from cohort 2 as described in Materials and Methods. Values are shown as means ± SEM, with the 95-percentile of the values shown in brackets underneath. The abbreviations are accordingly A1M (α_1-microglobulin) and Hb (hemoglobin).

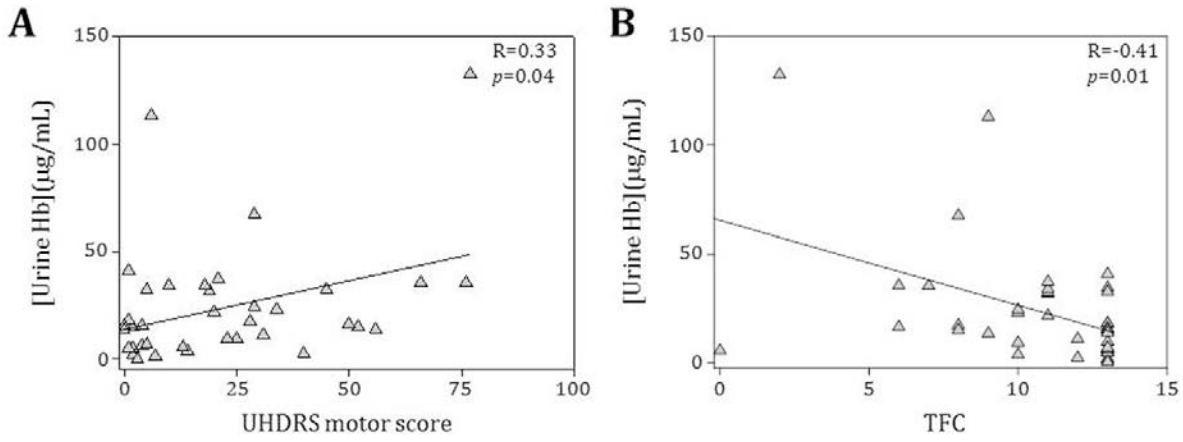

Figure 3. Correlations between urine Hb concentrations and clinical severity scores in premanifest and manifest HD gene carriers. Concentrations of Hb correlated with disease severity as demonstrated by increasing UHDRS motor scores (A) and decreasing total functional capacity score (B). Correlations with clinical variables were examined using Spearman´s correlations test.

significantly correlated to total functional capacity (TFC, p=0.004, r^2=0.20) and unified Huntington disease rating scale (UHDRS, p=0.02, r^2=0.14) (Figure 3). However, Hb levels were not seen to correlate with CAG repeat size. The levels of A1M, carbonyl groups or lipid peroxidation did not correlate with disease progression (as assessed by TFC and UHDRS scores).

The involvement of oxidation in HD was further investigated by analyzing levels of the lipid peroxidation product malonyldialdehyde in urine of all samples using the TBA assay (Figure 2D). The results displayed a trend towards an increase in the moderate group as compared to the control (moderate, mean value 0.058 ± 0.01; control, mean value 0.051 ± 0.01, One way ANOVA (Kruskal-Wallis test) p-value 0.33, post hoc Dunn´s test control vs. moderate p-value 0.66). Furthermore, a slight decrease was seen in the premanifest and early groups (premanifest, mean value 0.044 ± 0.01; early, mean value 0.042 ± 0.01).

4.3. Plasma markers of oxidation in cohort 2

The parameters measured in urine were also investigated in plasma of cohort 2 (Table 2). Although the same patterns of increased levels of Hb and A1M as in urine were observed, no significant elevations were seen. No obvious tendencies were seen in concentrations of protein carbonyl groups and lipid peroxidation.

5. DISCUSSION

Oxidative stress plays an important role in many neurological diseases, including Alzheimer's disease and Parkinson's disease (23). A large body of evidence support that, in both the human condition and in HD mice, oxidative stress may play a role in the pathogenesis of HD (3). Therefore, therapeutic strategies in order to reduce ROS may ameliorate the neurodegenerative process (24). Different laboratories have demonstrated oxidative damage in HD brains (25, 26) and there is evidence from both *in vivo* and *in vitro* studies showing oxidative damage to DNA, lipids and proteins (3).

Markers of oxidative damage have been suggested as possible biomarkers in neurodegenerative diseases. For example, 8-hydroxy-2' -deoxyguanosine (8-OHdG), a product of oxidative damage to DNA, has been suggested as a potential biomarker in Alzheimer's disease (27) and HD (28). However, relatively few studies have reported on such markers linked to oxidative stress in HD. Under oxidative stress conditions, proteins and lipids undergo a series of structural modifications that in many cases result in loss of function (19, 29). We have therefore investigated the levels of the oxidative markers protein carbonyl groups and lipid peroxidation in plasma and urine. In this material we were not able to detect any significant differences in either of the two markers. This could indicate that there is no increased systemic oxidation of proteins and membranes in our material.

However, it cannot be entirely excluded that other forms of oxidation occur, which could be measured by markers other than those assessed in this study.

In the present study, we detected significantly increased levels of Hb and A1M in HD urine samples compared with controls. The elevated urinary Hb-concentrations of early and moderate HD-patients may indicate an increased hemolysis in the patients. Mitochondrial dysfunction and metabolic abnormalities, which are implicated in HD pathology (3), have been shown to be involved in other diseases with a reported increased hemolysis, for example in preeclampsia (12, 30) and the HELLP syndrome (31). The mechanisms behind the hemolysis are still unknown, but the red blood cell destruction in these diseases, as well as in HD, may be induced by factors that arise as a result of increased metabolic activity. An attractive hypothesis is that free radicals and oxidative stress can modify erythrocyte membrane surface components, leading to a reduction in life-span and increased hemolysis. This has been shown to occur for instance during acute physical exercise (32) and was suggested as a pathological mechanism in diabetes (33, 34). However, it should be kept in mind that hemoglobinemia in preeclampsia and HELLP may have an entirely different etiology (e.g. due to hypertension and autoimmune processes) than HD.

It is generally accepted that Hb is a major generator of ROS when released in free form in plasma and at extravascular sites (5) and its downstream metabolites heme and free iron are potent inducers of oxidative damage (6). Although the levels of free Hb in the HD patients, demonstrated in this work, are lower than in for example sickle cell anemia (35) and preeclampsia (12) where they have been suggested to lead to pathological complications, a prolonged exposure to free, unprotected Hb may contribute to the oxidative stress observed in HD.

Elevated concentrations of A1M were seen in urine of HD patients of both cohorts. A1M is an important physiological scavenger of heme and radicals (8, 9). Its expression has been shown to be up-regulated in response to free Hb, heme and ROS (11). A1M is synthesized and secreted mostly from the liver, and rapidly distributed to different tissues where it is found in the extra-vascular compartments both in free form and as high molecular weight complexes bound to IgA, albumin and prothrombin (36, 37). A correlation between plasma concentrations of free Hb, oxidative stress markers and A1M were shown in pregnant women with preeclampsia at first and second trimester and at term (12, 38). A metabolical and biological link between Hb and A1M may be further supported by our current results, since these parameters were elevated in urine of HD patients.

Plasma Hb is filtered through the glomeruli of the kidneys and re-absorbed by the tubular epithelium where it may cause oxidative damage, including glomerular and tubular necrosis, during conditions with Hb overload (39). An impaired glomerular filtration and/or tubular re-absorption capacity may therefore be potential mechanisms that could explain the elevated urinary levels of Hb and A1M found in this work. However, no increase in the concentrations of albumin was found in any of the patient groups (not shown), suggesting that the kidney function is intact in the HD patients and that this is not a likely mechanism for the increased Hb and A1M-concentrations in urine. Alternative explanations may be increased plasma concentrations of Hb and A1M or a tubular synthesis of the proteins leading to excretion. Some support for the former mechanism was found from the analysis of free, extracellular Hb in plasma (Table 2). The increases in plasma were less pronounced than in urine and not statistically significant, but this is not surprising since the background levels of Hb in plasma of the control group were approximately 100-fold higher than in urine, possibly due to sampling-induced hemolysis. An increased hemolysis may therefore be masked by the higher normal levels of Hb in plasma.

Progress has been made towards developing panels of biomarkers (40), but there are no validated methods to assess underlying disease progression in gene carriers who have no overt disease signs (41, 42). A state marker, measurable in a readily accessible sample, a plasma or urine sample, would offer an aid in monitoring disease progression in future clinical trials. Urine-based proteomic profiling has shown promising results as a novel approach in the discovery of noninvasive biomarkers for diagnosing patients with different diseases, for both systemic and renal diseases (43). Urine has several advantages compared to for example blood plasma or cerebrospinal fluid, usually used for biomarker studies. Urine is easily accessible and does not need complex storage. Our results indicate that Hb in urine may be a possible biomarker associated with disease progression in HD. Future studies are warranted to investigate this further as well as validate results in longitudinal sample collections.

6. ACKNOWLEDGMENTS

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