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Forrest, C.M. and MacKay, G.M. and Stoy, N. and Spiden, S.L. and Taylor, R. and Stone, T.W. and Darlington, L.G. (2010) *Blood levels of kynurenines, interleukin-23 and soluble human leucocyte antigen-G at different stages of Huntington's disease*. *Journal of Neurochemistry*, 112 (1). pp. 112-122. ISSN 0022-3042

<http://eprints.gla.ac.uk/8995/>

Deposited on: 11 December 2009

**Blood levels of kynurenines, interleukin IL-23 and sHLA-G
at different stages of Huntington's disease**

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Running title: Kynurenines and Huntington's disease

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Abbreviations:- (continued.....)

Abbreviations:

EAE: experimental allergic encephalomyelitis;

3HAA: 3-hydroxyanthranilic acid;

3HK: 3-hydroxykynurenine;

HD: Huntington's disease;

sHLA-G: soluble Human Leucocyte Antigen-G;

IDO: indoleamine-2,3-dioxygenase;

IFN-: interferon- γ

IL-: interleukin-;

KMO: kynurenine-3-monoxygenase

NMDA: N-methyl-D-aspartate;

TLR: Toll-Like Receptor

Running title:

Kynurenines and Huntington's disease

Abstract

There is substantial evidence that abnormal concentrations of oxidised tryptophan metabolites, produced via the kynurenine pathway, contribute to progressive neurodegeneration in Huntington's disease. We have now examined the blood levels of these metabolites in patients at different stages of Huntington's disease, assessed both in terms of clinical disease severity and numbers of CAG repeats. Close relatives of the patients were included in the study as well as unrelated healthy controls. Levels of lipid peroxidation products, the pro-inflammatory cytokine interleukin-23 (IL-23) and the soluble human leucocyte antigen-G (sHLA-G) were also measured. There were lower levels of tryptophan and a higher kynurenine : tryptophan ratio, indicating activation of indoleamine-2,3-dioxygenase (IDO), in the most severely affected group of patients, with increased levels of IL-23 and sHLA-G. Marked correlations were noted between IL-23 and the patient severity group, anthranilic acid levels and the number of CAG repeats, and between anthranilic acid and IL-23, supporting our previous evidence of a relationship between anthranilic acid and inflammatory status. Tryptophan was negatively correlated with symptom severity and number of CAG repeats, and positively correlated with sHLA-G. The results support the proposal that tryptophan metabolism along the kynurenine pathway in Huntington's disease is related to the degree of genetic abnormality, to clinical disease severity and to aspects of immunopathogenesis.

Key-words: Huntington's disease; kynurenine; kynurenic acid; anthranilic acid; tryptophan IL-23, sHLA-G.

Introduction

Huntington's disease (HD) is an autosomal dominant disorder in which there is extensive degeneration of neurons in the striatum and neocortex. The clinical progression is variable but includes a movement disorder, cognitive decline and often a psychiatric component. Although a clear link has been made between the presence of the abnormal huntingtin protein, with an expanded series of glutamine residues close to its N-terminus (coded by CAG nucleotide triplet repeats) and the development of HD, the mechanisms by which this abnormality leads to neuronal loss remains unclear (Kuemmerle et al., 1999; Bates, 2003; Roze et al., 2008).

One possibility that has received much attention is that the kynurenine pathway (Figure 1), for the oxidative metabolism of tryptophan, contributes significantly to the neurodegeneration. This interest was originally focused on quinolinic acid, an endogenous tryptophan metabolite which is a selective agonist at N-methyl-D-aspartate (NMDA) receptors (Stone & Perkins, 1981; Stone, 2001; Stone & Darlington, 2002), with the resulting ability to induce excitotoxicity (Schwarcz et al., 1983). The administration of quinolinic acid into the striatum produces a pattern of cellular, neurochemical and behavioural changes closely resembling those seen in human HD (Beal et al., 1991a,b; Vecsei et al., 1991; Storey et al., 1994; Huang et al., 1995; Stone, 2001; Schwarcz et al., 2009). A role for glutamate, especially acting at NMDA receptors, continues to be strongly supported by evidence from patients and animal models (Cowan et al., 2008; Miller et al., 2008; Hassel et al., 2008; Heng et al., 2009).

However, other tryptophan metabolites in the kynurenine pathway are also likely to be relevant. Kynurenic acid is an antagonist at glutamate receptors (Perkins & Stone, 1982) and nicotinic cholinceptors (Hilmas et al., 2001; Stone, 2007), while 3-hydroxykynurenine (3HK) and 3-hydroxyanthranilic acid (3HAA) are redox active

compounds able to generate oxidative stress via the production of hydrogen peroxide under physiological conditions (Eastman & Guilarte, 1989, 1990; Nakagami et al., 1996; Okuda et al., 1996, 1998; Guidetti et al., 2000; Giles et al., 2003). Previous studies of the levels of these various tryptophan metabolites in HD has shown changes in several of them, supporting the view that the pathway may contribute to HD symptomatology. Kynurenic acid concentrations are lower in human post-mortem HD brain (Beal et al., 1992; Jauch et al., 1995), and an increased ratio of kynurenine to kynurenic acid has also been reported (Beal et al., 1990). There is increased enzymic activity of 3-hydroxyanthranilic acid oxygenase, which converts 3-hydroxyanthranilic acid into quinolinic acid in HD striatum (Schwarcz et al., 1988), with increased amounts of (3HK) - the precursor of 3HAA - in the putamen, frontal and temporal cortex of post mortem HD brains (Reynolds and Pearson, 1989; Pearson & Reynolds, 1992; Guidetti et al., 2000).

A previous analysis from this laboratory (Stoy et al., 2005c) showed that the kynurenine:tryptophan ratio was increased in the blood of HD patients compared with control subjects, indicating increased activity of the first enzymes of tryptophan oxidation: tryptophan-2,3-dioxygenase, found primarily in the liver or indoleamine-2,3-dioxygenase found in neurones, glia and immune system cells (IDO). These changes were accompanied by raised levels of inflammatory markers such as C-reactive protein, neopterin and lipid peroxidation products compared with controls. Changes were also demonstrated in the ratio of kynurenic acid:kynurenine, while levels of the redox-active 3HAA was decreased in HD patients, possibly as an adaptation to inflammation and increased oxidative stress. Overall, the results supported the view that tryptophan oxidation via the kynurenine pathway was altered in patients with HD.

That previous study, however, involved only hospitalised patients with advanced HD and we have now attempted to determine further the relevance of tryptophan metabolism by the kynurenine pathway by examining a series of patients at different stages of HD defined by the clinical assessment of symptom severity and by the length of the huntingtin gene CAG nucleotide triplet repeats.

Finally, there is growing interest in interleukin-23 (IL-23) and the soluble human leucocyte antigen-G (sHLA-G) in the expression of inflammatory conditions of the CNS, with the latter having a possible relationship to tryptophan metabolism (Gonzalez-Hernandez et al., 2005). We have, therefore, included measurements of these proteins in the full cohort of patients, relatives and controls.

Methods

Patients

It is well established that the length for CAG triplet expansion which is considered to be normal is up to around 36; in general subjects with 36 or fewer repeats have no evidence of brain pathology, motor or psychiatric abnormalities attributable to HD, while people with 40 or more repeats almost invariably have symptomatic evidence of the disorder. Patients with 37-39 repeats (reduced penetrance alleles) show reduced levels of symptoms. We have, therefore, defined patients as gene negative who had 36 or fewer CAG repeats and as gene positive if they had 37 or more repeats. The motor section of the Unified Huntington's Disease Rating Scale (The Huntington Study Group, 1986) was chosen as the most objective single measure of clinical disease severity currently available with the advantage that a score could be reliably established by a quick neurological examination. On this basis, participants were assigned to three clinical groups. The range of subjects

included those with no gene expansion and no overt clinical symptoms (group 0-, this group included subjects who were normal healthy volunteers working in Epsom General Hospital or associated laboratories and relatives of HD patients showing no gene expansion or symptoms), those with CAG expansion but no symptoms (0+), as well as patients with CAG expansions and mild-to-moderate disease severity (1+) or severe disease severity (2+). Inclusion of the 0- group allowed us to assess whether kynurenine pathway changes might occur in the relatives of patients with overt HD, even though those relatives had no discernible, accepted signs of the disease. The 0+ group should indicate whether the kynurenine pathway was affected in gene positive subjects independently of the existence of overt disease at the time of interview i.e. perhaps at an early stage of pathogenesis. The numbers of patients recruited to the different groups were as follows:- group 0-, n=29 (20f, 9m); group 0+, n=19 (14f, 5m); group 1+, n=14 (9f,5m); group 2+, n=40 (24f,16m), healthy controls n=11 (9f, 2m). None of the control subjects or patients reported any infections or illness within the previous 2 weeks before blood sampling and none showed any signs of infection at the time of sampling. Similarly none of the control subjects or patients had any psychiatric or neurological illness (other than HD) at the time of sampling.

Ethical approval for the study was obtained from the Ethical Committee of the Epsom and St Helier University Hospitals NHS Trust and all Research Governance requirements were achieved.

Blood sampling

Blood was collected by an experience phlebotomist from HD family members and controls in or near to their homes. Venous blood samples (25ml) were taken either into EDTA before being frozen in dry ice and transported to storage at -20°C

(for CAG repeats) or were allowed to clot at room temperature before being centrifuged and the serum removed for storage at -80°C until extraction and analysis.

CAG repeats

DNA was extracted from 4ml frozen blood in EDTA using the GeneCatcher™ automated blood kit by Invitrogen on the Tecan EVO150 liquid manual handling system in batches of 8 patients.

Sixty µl GeneCatcher™ magnetic beads and 12ml of 1 X GeneCatcher™ Lysis buffer (L12) were aspirated into eight 50ml falcon tubes and 4 sequential 1ml aliquots of patient blood added and mixed. The tubes were agitated at 300 rpm for 1 minute and incubated for 4 minutes at room temperature before discarding the supernatant. Ten ml of 1 X GeneCatcher™ Lysis buffer (L12) was added to each pellet before further agitation and removal of supernatant, and this process was repeated. Three ml of protease buffer and 40µl of protease were added to each tube and shaken to help resuspend the bead pellet after which the tubes were heated to 65-70°C for 5 minutes. The samples were mixed by trituration to disperse the bead/DNA pellet, incubated for another 10 minutes, and then 3ml of 100% isopropanol was added to each tube. After shaking at 400 rpm for 2 minutes until visible aggregates had formed, the tubes were heated once more and the supernatant discarded. Addition of 3ml of 50% (v/v) isopropanol was followed by further agitation, addition of 250µl of GeneCatcher™ Wash buffer (W12), supernatant removal and addition of 400µl of Elution buffer (E5) to each tube. After further rounds of shaking, incubation at 65-70°C and mixing by trituration, the supernatant containing the DNA was removed from the bead pellet and transferred to a sterile microcentrifuge tube, ready for further analysis.

PCR analysis

Testing for CAG repeat sequences was carried out by fluorescent PCR analysis using the HD1 and HD3 primers that flank the CAG repeat region in exon 1 of the *HD* gene (described by the HD collaborative research group 1993). The PCR was set up containing 0.025U Qiagen Taq with associated 1 X buffer (with magnesium) & Q solution, 0.25mM dNTPs (C, T, A, deaza-GTP), 0.5 μ M HD1 (dye-4 labelled) and HD3, 2 μ l patient DNA (1/10 dilution) in a total reaction volume of 20 μ l. The PCR was then amplified on a Hybaid PCR machine using the following parameters: 94°C for 4 minutes, then 30 cycles of 94°C for 45 seconds, 67.5°C for 45 seconds, 72°C for 45 seconds and a final step of 72°C for 10 minutes. For some samples where the HD1/3 PCR result was not conclusive/informative, a second PCR was performed using two alternative primers HD2 and HD4+ which are further out from the CAG repeat and incorporate an additional polymorphic CCG repeat as well as the CAG repeat in the PCR fragment. The CCG repeat is polymorphic and therefore, even when the CAG repeat is the same size for each allele, the PCR is likely to produce two different sized products. This PCR involved the substitution of HD2 (dye-3 labelled) and HD4+ in to the above PCR reaction conditions. The annealing temperature for the PCR reaction was also 63°C instead of 67.5°C. PCR primer sequences are as follows (5' to 3'):

HD1- : ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC

HD3- : GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC

HD2- : AAACACGGTCGGTGCAGCGGCTCCTCAG

HD4+: GCCATGGCGACCCTGGAAAAGC.

1 μ l PCR product were mixed with 40 μ l sample loading solution and 0.5 μ l 600PA size standard and run on a Beckman Coulter CEQ 8000 sequencer using Frag4 run module and auto-analysed using HD analysis parameters. Two positive

expansion controls and a negative control were also included in each run for quality control. In order for a result to be valid, all lanes need to have injected correctly, the size standard needs to have migrated the same in each lane, all peaks need to have a fluorescence of above 10,000 rfus, the positive controls need to give the expected results and the PCR negative control needs to have no peaks above 5% of those seen in the patient samples. The auto-analysis software automatically labels the one or two alleles present and determines the number of repeat units that the peak corresponds to (although this is also checked manually). This is done by calculating the size of the allele in base pairs, subtracting the constant region amplified by the primers (47bp) and then dividing the remaining bps by 3 to give the number of CAG repeats. This number is then increased by 2 to allow for a Beckman migration correction factor. The error margin of the assay method is ± 1 repeat. Exact allele sizes are not calculated for products of the HD2/HD4+ PCR, as two polymorphic regions are present in the PCR product.

Analysis of Kynurenines by HPLC

L-tryptophan and L-kynurenine were quantified using absorbance detection spectrometry, whereas kynurenic acid, 3-hydroxyanthranilic acid and anthranilic acid were quantified using fluorescence detection. The method used for tryptophan, kynurenine and kynurenic acid was based on that presented by Hervé *et al.* (1996) and the method for 3-hydroxyanthranilic acid and anthranilic acid was modified from that described by Cannazza *et al.* (2003).

During sample preparation, serum samples were kept on ice. To 500 μ l serum, 100 μ l of internal standard (240 μ M 3-nitro-L-tyrosine) and 50 μ l 2mM ascorbic acid were added, followed by 150 μ l 2M perchloric acid and 200 μ l water. Samples were shaken and then centrifuged at 5000g for 10 minutes at 4°C. The supernatants were

collected and filtered using centrifugation at 10,000g for 10 minutes at 4°C, prior to injection of 100µl onto the HPLC column.

Isocratic reversed-phase HPLC was performed at 37°C, using a Waters HPLC system. For tryptophan, kynurenine and kynurenic acid determination, separation was achieved using a Synergi Hydro column (250 x 4.6mm I.D., particle size 4µm; Phenomenex, Macclesfield, Cheshire, UK), with detection by a Waters 2487 dual wavelength absorbance detector and a Waters 474 fluorescence detector, connected in series. Tryptophan was determined by absorption spectrometry at 250nm and kynurenine at 365nm. The internal standard, 3-nitrotyrosine, was quantified at 365nm. Kynurenic acid was determined by fluorescence detection (excitation 344nm, emission 390nm). The mobile phase was 50mM acetic acid, 100mM zinc acetate containing 1.5% acetonitrile using a flow rate of 1ml/min. Zinc acetate enhances the fluorescence of kynurenic acid (Shibata, 1988).

3-hydroxyanthranilic acid and anthranilic acid were separated using a Synergi Hydro column (250 x 4.6mm I.D., particle size 4µm, Phenomenex) and fluorescence detection (excitation 320nm, emission 420nm). The mobile phase was 25mM sodium acetate buffer at pH5.5, at a flow rate of 1ml/min.

The limits of detection using an injection volume of 100µl and a signal-to-noise threshold of 3, were: tryptophan 20pmoles, kynurenine 1pmole, kynurenic acid 0.2pmoles, 3-hydroxyanthranilic acid 10fmoles, and anthranilic acid 20fmoles on column. Recoveries determined using blood samples spiked with standards of the measured compounds were between 90 and 110% for tryptophan, kynurenine, kynurenic acid, 3-hydroxyanthranilic acid and anthranilic acid.

IL-23

Levels of IL-23 were determined in serum using a commercially available Biosource human IL-23 immunoassay kit (Invitrogen, Paisley, UK). This kit detected the human IL-23 heterodimeric protein consisting of subunits p19 and p40, and did not recognise the monomeric forms of the p19 and p40 subunits.

sHLA-G

The concentration of soluble HLA-G was measured in serum samples using an ExBIO/BioVendor ELISA assay kit supplied by AXXORA (Nottingham, UK). The assay was specific for the HLA-G1 and HLA-G5 isoforms.

Lipid peroxidation

The lipid peroxidation products malondialdehyde and 4-hydroxynonenal were measured in serum using a Bioxytech LPO-586 colorimetric assay (Biostat, Stockport, UK). The analysis involved the reaction of N-methyl-2-phenylindole with these peroxidation products to form a stable chromogenic indolic dimer which was detected spectrophotometrically at 595nm using a microplate reader.

Statistics

All data are presented as the mean \pm 1 standard error of the mean. Ordinary analysis of variance (ANOVA) followed by Dunnett's post-test was carried out to compare healthy controls with each of the HD patient groups. Where the data was non-parametric ANOVA was followed by Dunn's post-test. Correlations between variables were obtained using a correlation matrix which generated the Spearman correlation coefficients and level of statistical significance. In all cases a significance threshold of 5% ($p < 0.05$) was employed.

Results

Kynurenines

The level of tryptophan in the serum shows a trend to decrease as the severity of HD increased (Fig. 2A). This became significant in the 2+ group of patients who are both gene positive and exhibit the most severe symptoms. Even though there was no similar, significant change in the absolute levels of kynurenine (Fig. 2B), the ratio between the substrate and product of IDO activity (the k:t ratio) was altered significantly in the 2+ patients (Fig. 2C).

There were no significant changes in the levels of kynurenines beyond kynurenine itself, including kynurenic acid, which represents the product of activity in the kynurenine aminotransferase enzymes (Fig. 3A), 3-hydroxyanthranilic acid (3HAA) which is the product of kynurenine mono-oxygenase and kynureninase activity (Fig. 3B), or anthranilic acid which is produced solely by kynureninase activity (Fig. 3C).

Kynurenine correlations

The use of absolute values across patient groups is dependent on the means and variances of the populations assessed and can mask specific relationships between individual parameters across the population. Correlations generate more useful information about the relationships between factors in individual patients. In this case, there were clear and highly significant negative correlations between the levels of tryptophan and the patient group (0-, 0+, 1+ or 2+) (Table 1), and between tryptophan levels and CAG repeat length (Table 1). These were all negative correlations suggesting a relationship with the activation of tryptophan metabolism by

IDO, this possibility being supported by the strong positive correlations between k:t ratio and group, age and CAG repeats. In contrast, there were no significant correlations between kynurenine levels and patient group or CAG repeats (Table 1).

The relationship with age could represent a cause or a consequence of the presence of disease. Indeed, several groups have noted similar correlations between tryptophan, k:t ratios or kynurenic acid levels and age (Kepplinger et al., 2005; Pertovaara et al., 2006). Indeed, one of the difficulties in studies of neurodegenerative disorders is their tendency to be age-related. However, it is very unlikely that the changes observed in the present study are attributable primarily to age, for three reasons. Firstly, tryptophan levels were not correlated with age in our patient population (Table 1). Secondly, there was little difference in the age distribution of patients across the classification groups (Fig. 4). An analysis of variance revealed a significant difference across the groups ($F(4,112)=8.96$, $p < 0.0001$), but only the mean age of the 0+ group of patients was significantly different from the control group ($p < 0.05$, Dunnett's test). Thirdly, even if the relationship between kynurenines and disease severity was caused by the progressive worsening of symptoms with age, the measurement of CAG repeat length was performed in parallel on the same blood samples used for the other analyses and age cannot, therefore, account for the genetic correlations observed. There was no correlation between CAG repeats and age ($r = 0.10$, $p = 0.27$).

Finally there were several correlations between tryptophan metabolites and inflammatory markers (see below and Table 1).

Inflammatory markers

Although the absolute levels of lipid peroxidation products, which are commonly used to reflect the degree of oxidative stress in patients, were not

changed in these patients (Fig. 5A), there were significant differences in both IL-23 (Fig. 5B) and sHLA-G (Fig. 5C). For both of these compounds, there was a clear trend to an increase in absolute values with increasing severity of the disease, with significant differences appearing in the most severely affected subjects.

Inflammatory marker correlations

IL-23 levels correlated with the overall patient classification group of disease severity ($r = 0.414$, $p = 0.0006$), age ($r = 0.288$, $p = 0.0022$) and the number of CAG repeats ($r = 0.283$, $p = 0.0026$). The levels also correlated with the k:t ratio, levels of kynurenic acid and levels of anthranilic acid (Table 1). Levels of tryptophan were correlated with sHLA-G.

Discussion

Since the first reports by Beal et al., (1986, 1991a,b) that intrastriatal administration of quinolinic acid to rodents produced a pattern of neurotransmitter and neuropeptide changes closely similar to that seen in human post-mortem HD tissue, there has been a growing interest in the role of this compound and other tryptophan metabolites in the disease (Stone, 1993, 2001). Indeed, quinolinic acid lesions of the striatum have become widely accepted as an experimental model for HD (Schwarz et al., 1992; Popoli et al., 1994; Hughes et al., 1999). The fidelity of the quinolinic acid model is increased by localised administration into the posterior putamen of non-human primates (Burns et al., 1995).

There is now strong evidence that an over-expression of the abnormal huntingtin gene contributes to the induction of HD symptoms in humans and to the striatal characteristics of HD in mouse models (Carter et al., 1999; Sathasivam et al.,

1999; Usdin et al., 1999), so it is intriguing that administration of quinolinic acid into the striatum can induce an increased expression of huntingtin (Tatter et al., 1995), raising questions about which is causal and which is effect. Perhaps a primary toxicity caused by kynurenine metabolites could induce huntingtin as the cytoprotective response postulated by some authors (Kuemmerle et al. 1999; Bates, 2003).

Interest in the kynurenine pathway has been strengthened by evidence that levels of kynurenic acid and the redox-active metabolite 3HK are also altered in HD brain compared with controls (Reynolds and Pearson, 1989; Pearson and Reynolds, 1992; Guidetti et al., 2000). The former change adds to the possibility that the degree of activation of NMDA receptors (by glutamate or quinolinic acid) and their blockade by kynurenic acid could contribute to the development of HD, while the changes of 3HK would be consistent with increased oxidative stress in the disorder which has been proposed by several authors (Browne et al., 1999; Tabrizi et al., 2000; Stoy et al., 2005c; Perez-de la Cruz et al., 2009). Altered levels of kynurenic acid and 3HK can also be demonstrated in early stage patients or mouse mutants over-expressing the huntingtin protein (Guidetti et al., 2000).

In addition, recent microarray work (Giorgini et al., 2005) on a yeast model engineered to over-express huntingtin protein revealed that the most marked molecular change induced was an increase in the expression of kynurenine-3-monooxygenase (KMO), the enzyme promoting the conversion of kynurenine into 3HK and, thus, to 3HAA and quinolinic acid.

Although the use of peripheral measurements of compounds to reflect activity within the CNS is fraught with difficulties, since distinguishing central from peripheral sources is difficult to achieve, it has been possible to show changes in tryptophan metabolites that support direct measurements of their levels in post-mortem brain or

animal models (Stoy et al., 2005c). In addition, parallel studies of cytokine levels in blood and CSF have shown strictly comparable changes, leading to the suggestion that the genetic alteration in huntingtin expression - known to occur in peripheral tissues as well as the CNS – is associated with activation of both peripheral monocytes/macrophages and central microglia (Sapp et al., 2001; Bjorkqvist et al., 2008). One consequence of this is that blood measurements are likely to reflect the same qualitative changes occurring in the CNS, even if the compounds measured are of peripheral origin. However, this assertion needs to be qualified by emphasising that only some components of the kynurenine pathway – such as tryptophan itself and kynurenine - cross the blood-brain barrier readily using the large neutral amino acid transporter (Fukui et al., 1991; Vecsei et al., 1992, Jauch et al., 1993), whereas kynurenic acid and 3HAA cross poorly by diffusion alone.

An increased k:t ratio suggests a degree of immune activation in the HD patients. This is consistent with a previous report by Leblhuber et al., (1998) in which patients with HD were shown to have increased levels of inflammatory markers such as neopterin, immunoglobulin IgA, soluble receptor for tumour necrosis factor- α (sTNF-R) and the IL-2 receptor sIL-2R. These changes were associated with a significant reduction in blood tryptophan levels consistent with an activation of IDO, a finding confirmed in the present study. In addition, the presence of an altered inflammatory state in HD is supported by recent data from Bjorkqvist et al., (2008) showing raised levels of several cytokines in both plasma and CSF. Their data are consistent with the view that innate immune cells are autonomously activated either by intrinsic huntingtin-related abnormalities and/or by alterations in immune responsiveness to toll-like receptors (TLRs) or other receptor inputs, resulting in a disturbed balance of pro- and anti-inflammatory cytokines in the blood and CSF characteristic of chronic inflammatory conditions (Stoy 2005a,b; Bunt et al., 2009).

The most sensitive blood marker of presymptomatic HD was IL-6 (Bjorkqvist et al. 2008), elevated up to a mean of 16 years before first clinical disease manifestations. Against this background we chose to measure the levels of IL-23, which is an innate pro-inflammatory member of the IL-12 family, that induces IL-17 from a subset of T cells (Th-17). We found that there was a significant correlation between levels of IL-23 and both the concentration of kynurenic acid and the k:t ratio (signifying kynurenine pathway activation) as well as anthranilic acid, which we have previously noted to be increased in the presence of inflammation (Forrest et al., 2006) or brain damage (Mackay et al., 2006; Darlington et al., 2007).

Although IL-6 is considered important for commitment to the Th17 lineage, IL-17 output of memory T cells is primarily sustained by IL-23. Both CD4+ and CD8+ T cells can express IL-23 receptors. Given the known immuno-suppressive properties of IDO, it is noteworthy that in murine collagen-induced arthritis, IDO upregulation occurs in lymph node dendritic cells and is associated with decreased production of IL-17 and IFN- γ from Th-17 and Th1 cells that subsequently become located in inflamed joints (Criado 2009). A recent study has demonstrated probable constitutive expression of functional IL-17 receptors on murine microglia, and to a lesser extent astrocytes, and these respond actively to IL-17, as evidenced by the induction of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2. With respect to HD, there is accumulating evidence that huntingtin-derived N-terminal oligomers trigger microglial activation through receptors of the innate immune system (Haass and Selkoe, 2007; Thakur et al., 2009). It is interesting to speculate that the activation profile of such microglia might include upregulation of IL-23 alongside IL-6 and IL-8.

The view that immune activation is involved in HD pathogenesis is further expanded by our data on sHLA-G. A wide range of values for sHLA-G were found in

the subjects tested here, ranging from undetectable to very high levels of around 100U/ml. A similar degree of variation has been described by other groups (Hunt et al., 2000). Despite this variation, there was a trend for the levels to increase with disease severity and huntingtin gene expansion (Fig. 5C), with the changes being statistically significant in the most severely affected group of patients.

sHLA-G has potent immunosuppressive properties. It is present in brain and CSF in multiple sclerosis, in which it correlates positively with remission and IFN- β therapy (Wiendl et al., 2005; Fainardi et al., 2006), and has recently been found in blood and CSF in several other inflammatory conditions of the CNS (Feger et al. 2007; Wiendl, 2007). It induces apoptosis of activated CD8(+) T cells and inhibits proliferation of CD4(+) T cells and may play a role in the suppression of tumour growth and T cell activity associated with a maternal immune system attack on developing allogeneic embryos (Moreau et al., 1998; Fuzzi et al., 2002; Carosella et al., 2003a,b). It is known that a similar function attaches to the activation of IDO. Enzyme activity in the pregnant mother prevents abortion of allogeneic fetuses, while inhibition of IDO leads to the loss of embryos (Munn et al., 1998). It could be, therefore, that there is a close mechanistic relationship between the activation of IDO and the expression of sHLA-G. A recent review (Pistoia et al., 2007) noted the uncertainty about whether sHLA-G levels were related to other immune system markers and about the factors which regulated the levels of sHLA-G in tissue fluids. This suggestion is entirely consistent with evidence that the activation of IDO may play a role in the expression of sHLA-G. Both kynurenine (Lopez et al., 2006) and 3HAA (Lopez et al., 2008) can increase the expression of sHLA-G in macrophages, (although kynurenine might act indirectly after its conversion to 3HAA). Alternatively, since sHLA-G has been shown to induce the expression and release of IFN- γ from blood mononuclear cells (Kanai et al., 2001), it may be that sHLA-G indirectly (via

IFN- γ) produces the activation of IDO and, thus, kynurenine production.

Furthermore, under various conditions of immunostimulation or immunosuppression (autoimmunity, allergy, cancer) IFN- α , IFN- β , IFN- γ or IL-10 have all been shown to increase the release of sHLA-G from peripheral blood monocytes (and by implication microglia), suggesting that sHLA-G might be acting rather like an innate immune system 'rheostat' (Borghesi et al., 2008, Mapp et al., 2009, Rebmann V et al., 2003). There is clearly potential here for the emergence of feedback loops with a major influence on immunological and neuronal function.

It has also been noted that IDO activity can modify the expression of sHLA-G (Gonzalez-Hernandez et al., 2005), and the existence of a relationship is supported by our finding correlations between this antigen and levels of tryptophan (Table 1). There is evidence that sHLA-G and IDO inhibit T-cell proliferation by independent molecular pathways (Le Rond et al., 2005), and that neither the antigen nor tryptophan and its metabolites are able to modulate the activities of the other system. In addition, the interactions noted by Gonzalez-Hernandez et al., (2005) appeared to be related to IDO activity independently of its ability to oxidise tryptophan along the kynurenine pathway.

Overall, the correlations between HD parameters and the kynurenine metabolites are consistent with a role for these compounds in HD. The secretion of cytokines induced by damaged neurons expressing mutant huntingtin can produce microglial activation, including IDO, with the production of 3HK and quinolinic acid which could then contribute to further damage (Schwarcz et al., 2002). In yeast cells expressing an abnormal expanded huntingtin protein, inhibiting the kynurenine pathway reduced poly-Q-mediated toxicity as well as the generation of 3-HK, quinolinic acid and reactive radicals. Furthermore, a high-throughput screen of compounds using the yeast model led to the identification of a molecule which

prevented neurodegeneration induced by polyQ (Zhang et al., 2005). The active compound proved to be an analogue of the KMO inhibitor Ro61-8048, and the results suggest that the inhibition of KMO may counteract huntingtin-mediated toxicity.

In conclusion, our data suggests that the kynurenine pathway may be associated with the pathogenesis of HD. We have also found correlations involving kynurenines, IL-23, sHLA-G and disease severity. Taken together, our data supports the emerging view that the balance of neurodegeneration and neuroprotection is not determined purely by neuronally autonomous mechanisms but is significantly impacted by a network of peripherally and centrally acting cytokines and signalling molecules that modulate inflammatory and immune responses. More detailed analysis of this network in HD model systems and further investigation of KMO and perhaps other enzymes in the kynurenine pathway may well reveal new drug targets for the treatment of Huntington's disease.

Acknowledgements

We wish to thank the many representatives of the Huntington's Disease Society, both centrally and throughout the U.K., for their help and support and we thank their members most sincerely for agreeing to participate in the study. We also thank Mrs Valerie Cox, Ms. Gillian Harman and Ms. Sue Baker for travelling to meet the patients, their family members and controls to obtain the necessary blood samples. Without the teamwork of all these people the study could not have been completed. We are grateful to Epsom Medical Research and the Peacock Trust for financial support.

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Figure 1

A schematic summary of the main components of the kynurenine pathway for the oxidation of tryptophan.

Figure 2

Levels of tryptophan, kynurenine and the kynurenine:tryptophan ratio (k:t ratio) in control subjects or patients at different stages of Huntington's disease. The columns indicate the mean \pm s.e.mean for subjects with no gene expansion and no overt clinical symptoms (group 0-, controls and relatives of HD patients), those with CAG expansion but no symptoms (0+), as well as patients with CAG expansions and mild-to-moderate disease symptoms (1+) or severe symptoms (2+).

* $p < 0.05$, ** $p < 0.01$ relative to the control group.

Figure 3

Levels of kynurenic acid, 3-hydroxyanthranilic acid (3HAA) and anthranilic acid (AA) in control subjects or patients at different stages of Huntington's disease. The columns indicate the mean \pm s.e.mean for subjects with no gene expansion and no overt clinical symptoms (group 0-, controls and relatives of HD patients), those with CAG expansion but no symptoms (0+), as well as patients with CAG expansions and mild-to-moderate disease symptoms (1+) or severe symptoms (2+).

Figure 4

Bar chart of the age distribution of the subjects examined in this study. There were no significant differences in the age of subjects in the various categories compared with the control group.

Figure 5

Levels of lipid peroxidation products, IL-23 and sHLA-G in control subjects or patients at different stages of Huntington's disease. The columns indicate the mean \pm s.e.mean for subjects with no gene expansion and no overt clinical symptoms (group 0-, controls and relatives of HD patients), those with CAG expansion but no symptoms (0+), as well as patients with CAG expansions and mild-to-moderate disease symptoms (1+) or severe symptoms (2+).

* $p < 0.05$, ** $p < 0.01$ relative to the control group.

Table 1

Significant correlations between levels of tryptophan metabolites and clinical parameters, showing Spearman correlation coefficient and p value where significant.

	Symptom group	Age	CAG length	IL-23	SHLA-G
Tryptophan	- 0.427 p=0.000002	-	- 0.325 p=0.00043	-	0.224 p=0.017
Kynurenine	-	0.405 p=0.000009	-	-	-
K:T ratio	0.354 p=0.0001	0.434 p=0.000001	0.281 p=0.0025	0.190 p=0.046	-
Kynurenic acid	-	-	-	0.191 p=0.045	-
3HAA	-	-	-	-	-
AA	-	0.275 p=0.0032	-	0.192 p=0.043	-

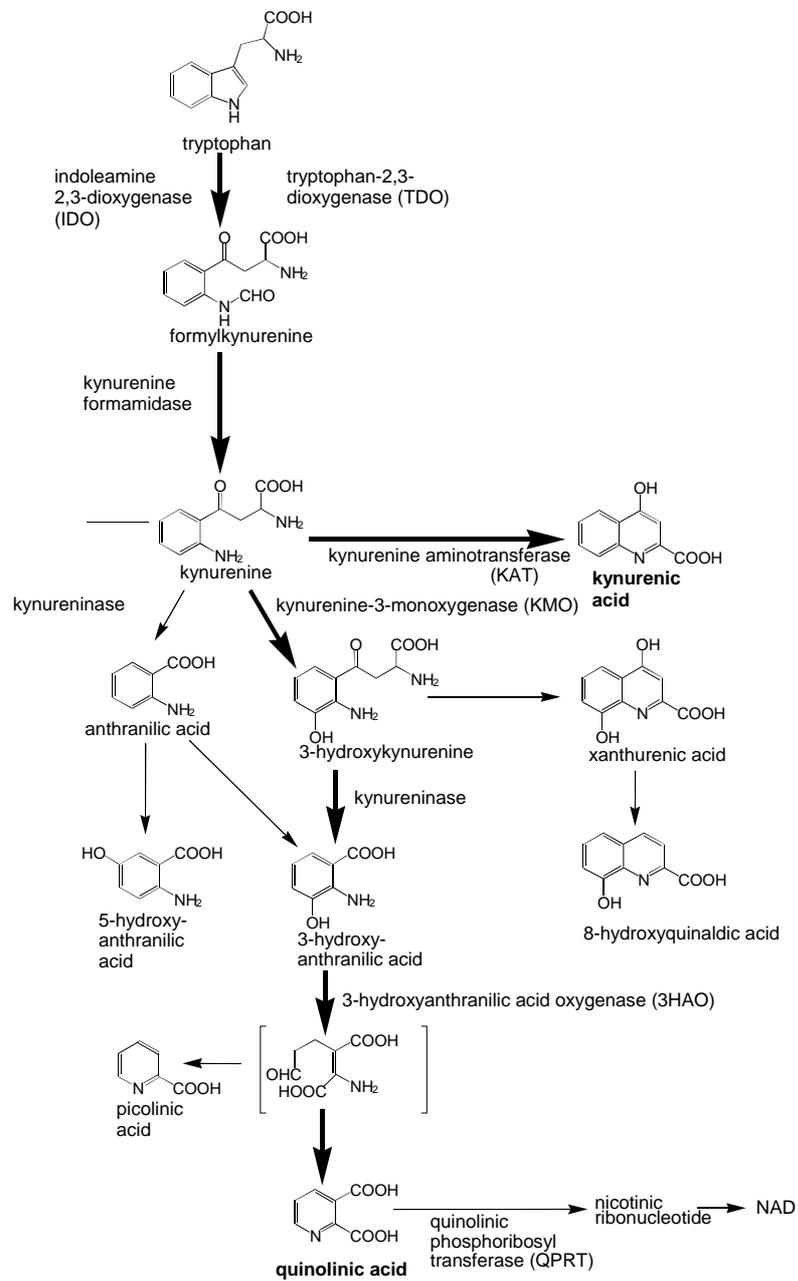


Fig. 1

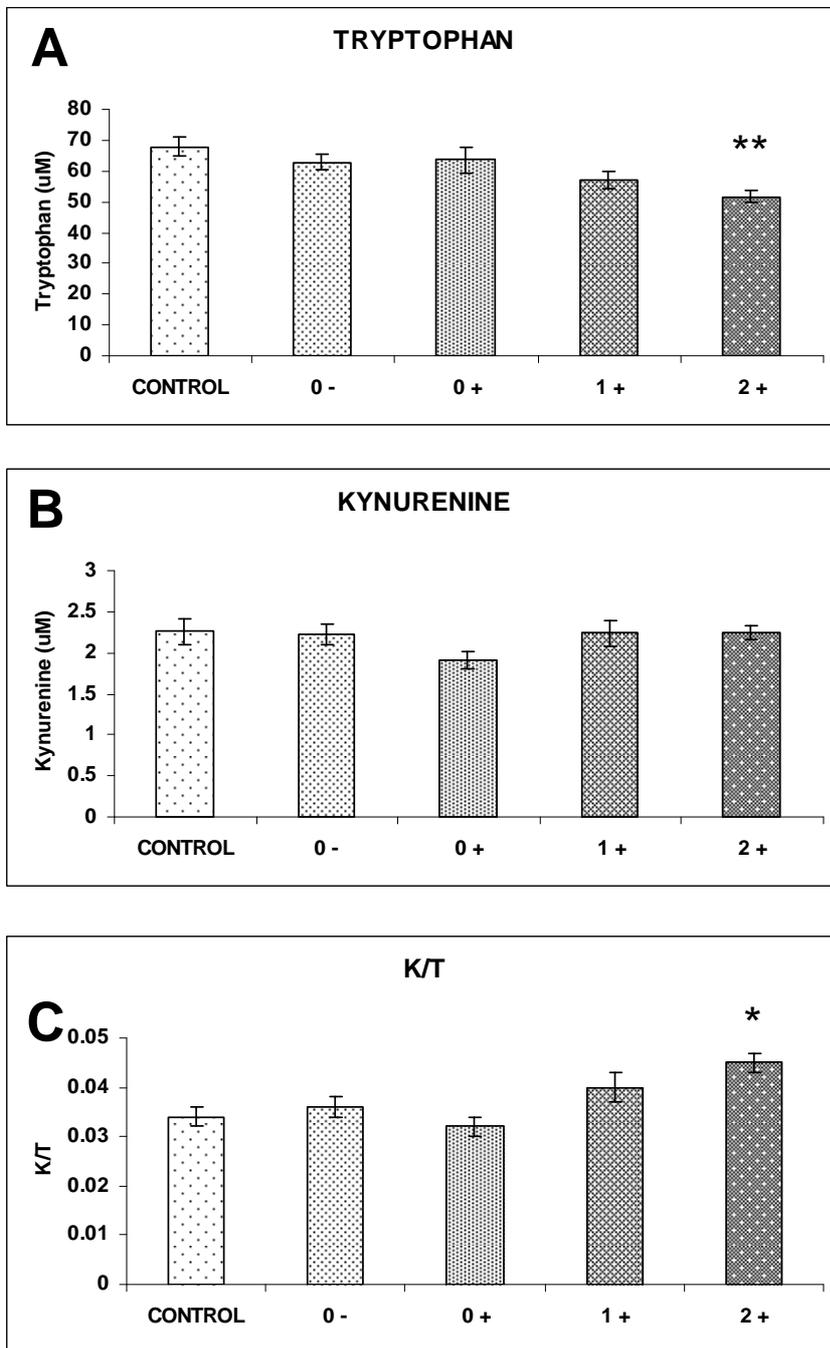


Fig. 2

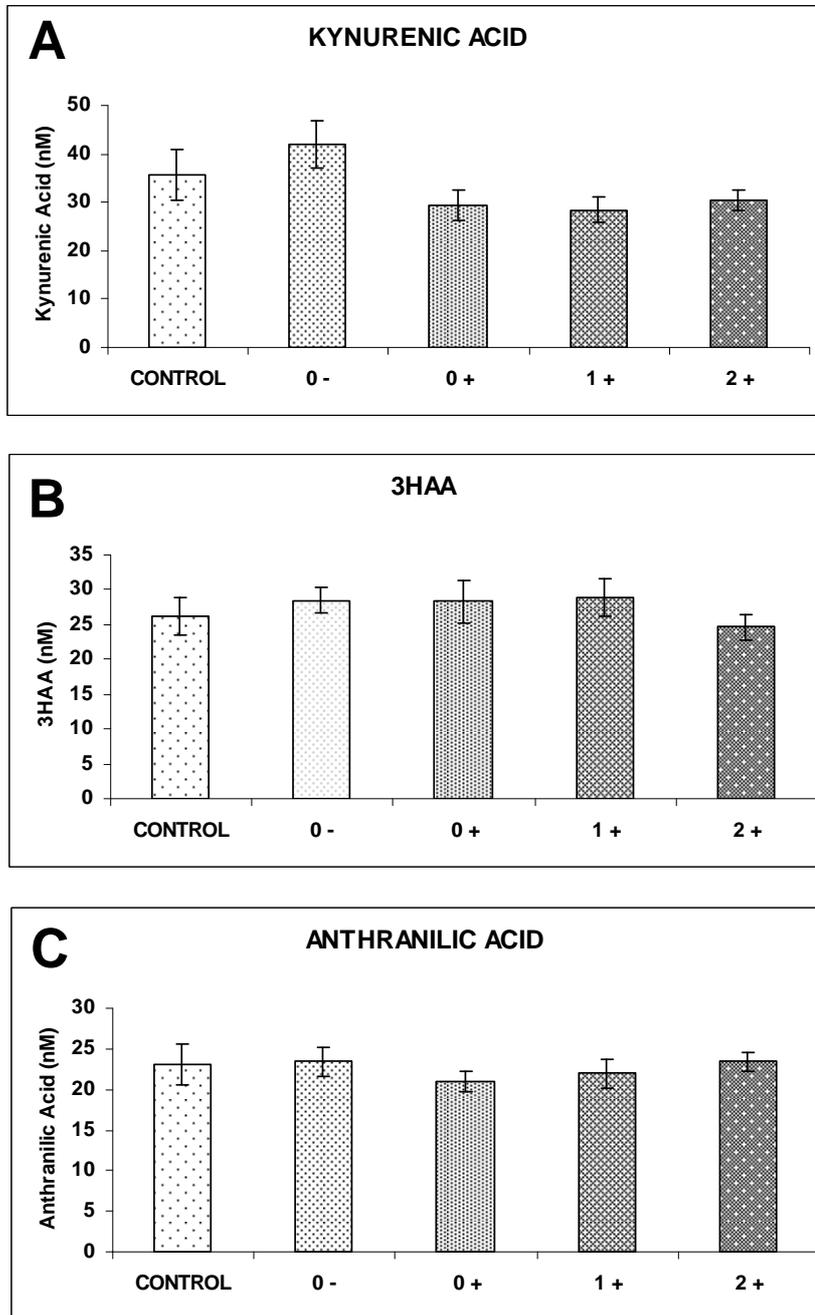


Fig. 3

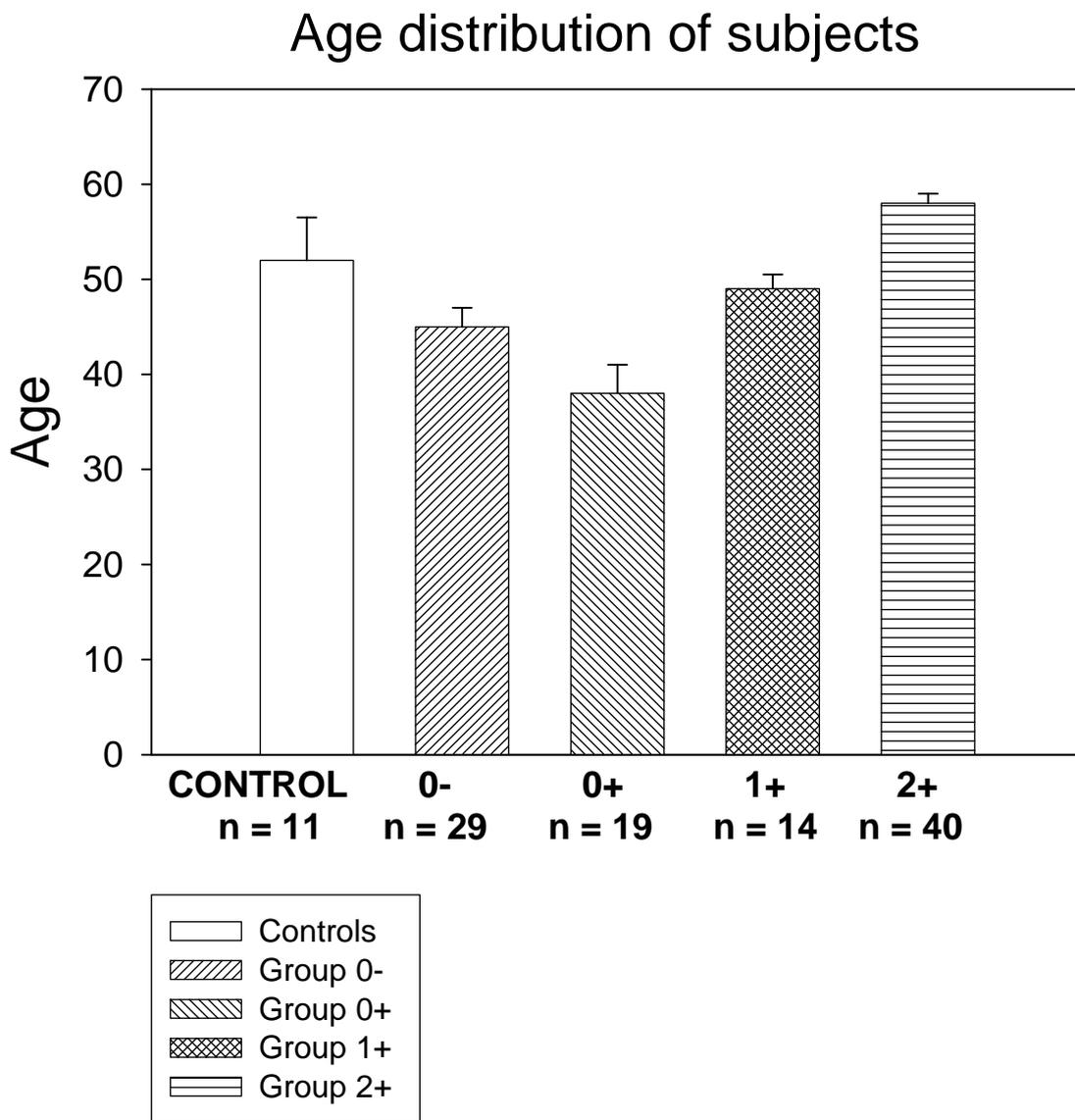


Fig. 4

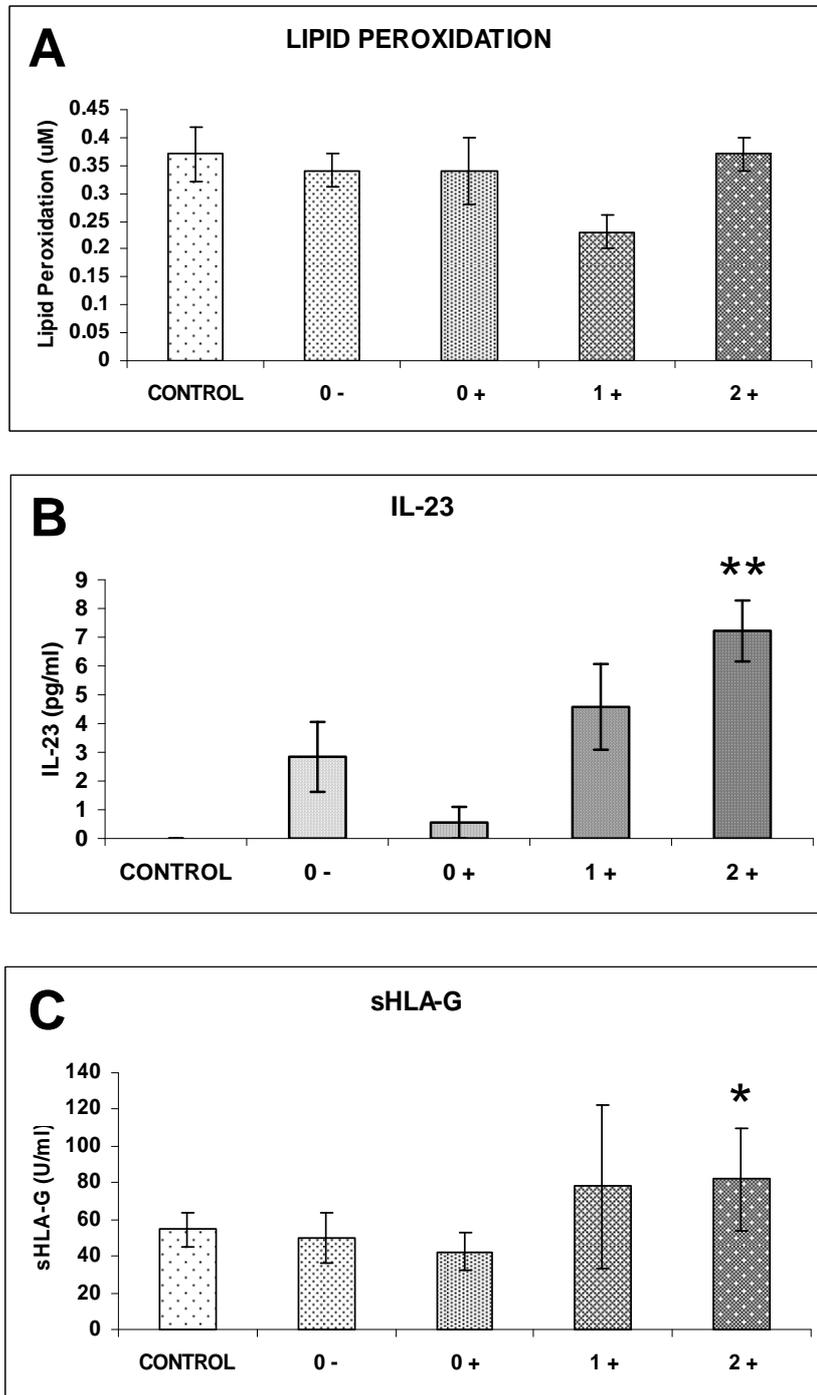


Fig. 5