

# **A1M, Hb and tryptophan metabolism in R6/2 mouse model of Huntington's disease**

## **Huntington's disease**

Huntington's disease (HD) is a genetic disorder characterized by selective neuronal loss in the striatum, leading to deterioration of motor function and involuntary movements, progressive psychiatric symptoms, cognitive impairment and eventually a premature death.

HD is inherited in an autosomal dominant way. Since the 1990s it has been known that the disease is caused by a chromosomal mutation leading to the expression of a mutant protein, mutant Huntingtin (mHtt). The physiological role of Huntingtin is not fully known, but mHtt, is expressed in cells throughout the entire body (*Soulet and Cicchetti 2011, Ross & Tabrizi 2011*).

In accordance with its most prominent symptoms, HD has primarily been seen as a disease of the brain. However, HD has several systemic manifestations such as weight loss, muscular atrophy, impaired glucose tolerance and hypercortisolism (*Soulet and Cicchetti 2011*). Also, recent findings suggest that mHtt interacts directly with the immune system and changes in the immune system may critically contribute to the pathology of HD (*Soulet and Cicchetti 2011*). An altered immune profile is seen even in premanifest HD patients (*Björkvist et al 2008*).

## **Tryptophan, the immune system and the brain**

In mammals, approximately one percent of the amino acid tryptophan (trp) is used as a precursor of the well known neurotransmitters serotonin (5-HT) and melatonin. However, 95 percent of trp is metabolized via the so called kynurenine pathway (KP) (eventually resulting in the production of NAD) (Fig 1). An increased or altered flux through this pathway is seen in numerous pathological conditions associated with impaired brain functioning, such as HD, Alzheimer's disease (AD), the AIDS dementia complex, depression and schizophrenia (*Chen and Guillemin 2009, Stone et al 2011, Zádori et al 2009*).

A key enzyme in the KP is IDO (Indoleamine 2,3-dioxygenase). IDO has been widely studied regarding its role in immunity, as it is involved in immunoregulation and is up-regulated by inflammatory cytokines, mainly IFN  $\gamma$  (*O'Connor 2009, Guillemin 2004*). Activation of IDO leads to an increased formation of kynurenine (kyn). IDO is inducible in most cells (*King and Thomas 2007*), and increased expression in brain has been seen in AD (*Bonda et al 2010*). The enzyme was not detected in healthy human brain cells *in vitro*, but was readily induced by addition of IFN- $\gamma$  to the medium (*Guillemin et al 2004*).

Trp metabolism is also regulated by the hepatic enzyme TDO (Tryptophan 2,3-dioxygenase). TDO is expressed constitutively and is primarily regulated by availability of trp, but can also be upregulated by glucocorticoids (*Oxenkrug 2012, Young 1981*)

### **Neurotoxic tryptophan metabolites in HD**

The metabolism of kyn produces a number of compounds that are neuroactive. However, several metabolites are also neurotoxic. In 2010 *Sathyasaikumar et al* described increased production and decreased degradation of 3-hydroxykynurenin (3-HK) in brains of R6/2 mice, a well studied animal model of HD. Further experiments linked this directly to neuronal damage. Elevated levels of 3-HK have also been seen in post mortem examinations of brains from HD patients (*Guidetti et al 2004*). 3-HK damages brain cells through the formation of reactive oxygen species (ROS) and by augmenting the toxicity of further downstream metabolites such as quinolinic acid<sup>1</sup> (*Okuda et al 1996, Stone 2001*) (Fig 1). The production of 3-HK takes place mainly in microglia, the resident macrophages of the brain, which are pathologically activated even in pre-manifest HD patients (*Politi et al 2011*).

HD patients have decreased plasma trp and an increase in kyn/trp ratio (*Stoy et al 2005*) but it is not known whether there is a peripheral increase in kyn production or if there is for example a local up-regulation of IDO in brain. Kyn can be produced in the CNS as a result of local IDO activation (*Kwidzinski and Bechmann 2007*) but also transported across the blood-brain barrier from peripheral production sites, for example, after IDO-activation in a systemic inflammatory challenge (*Fukui*

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<sup>1</sup> The original interest in trp metabolism in HD started with the discovery that rats who were given intrastriatal injections of quinolinic acid developed HD-like symptoms and the same type of neuronal degeneration.

1991). This has been proposed as a pathogenetic mechanism behind neuropsychiatric symptoms in uremic patients (*Topscevska-Bruns* 2008) and also in major depression (*Maes* 2010).

### **Alpha-1-microglobulin and 3-HK**

Alpha-1-microglobulin (A1M) is a ubiquitous low molecular weight plasma and tissue protein with radical scavenging abilities. As described below it may also be a natural scavenger of 3-HK and its autoxidation products. A1M is mainly synthesized in the liver, but also in lesser amounts in peripheral organs such as blood cells, skin, pancreas and kidney. Synthesis is increased in liver and locally in response to increased levels of ROS and free heme-groups. A1M is proposed to act as a “radical sink”, trapping ROS and radicals by covalent binding. When the molecule is saturated by covalently bound radicals it is cleared from tissues by the blood and subsequent glomerular filtration in the kidneys (*Åkerström* 2007, *Olsson et al* 2012).

In 2004 *Sala et al* found that A1M isolated from urines of patients undergoing hemodialysis was covalently bound to 3-HK. Tryptophan catabolism via the KP is increased in uremia and plasma levels of 3-HK can be elevated threefold in end stage renal failure as compared to controls (*Pawlak et al* 2008). Elevated levels of 3-HK are also found in brain in experimental animal models of chronic renal insufficiency (*Topczewska-Bruns et al* 2002), in analogy with the situation in HD.

### **Hemoglobin**

A previous study (*Olsson et al* 2012) showed that urine and plasma levels of hemoglobin (Hb) were elevated in HD-patients, and that Hb levels in urine correlated with disease severity. The findings were reproduced in two separate cohorts of HD patients (N= 130, N=96). In the study, plasma and urine levels of A1M were elevated in HD patients as compared to controls, although A1M levels did not correlate with disease severity. Due to oxidation/reduction-reactions of Hb and its metabolites heme and iron, free Hb released from red blood cells by hemolysis induces inflammation, oxidative stress and production of ROS (*Everse and Hsia* 1997, *Olsson et al* 2012). Free Hb has also, interestingly, been seen to up-regulate IDO in dendritic cells. This was seen by *Ogasawara et al* (2009) after the discovery that patients suffering from hemolytic anemia had a significantly decreased kyn/trp ratio.

## **Hypothesis and aim**

We hypothesized that A1M is up-regulated by the elevated levels of 3-HK and Hb in HD, and thus mobilized as a natural defense mechanism against 3-HK and Hb-induced oxidative stress in the disease. The success/failure of the A1M defense mechanism may have a role in the clinical development of HD in vivo.

To test this hypothesis, we have measured the expression of the IDO-, TDO- and A1M-genes in brain, liver and kidneys, and the concentrations of A1M and Hb in plasma, of R6/2 and healthy mice.

## **Methods and materials**

### **Animals and sample collection**

The R6/2 line is a well studied model in HD and was created by inserting a 1,9-kb genomic fragment of the human htt promoter and exon 1 of the human huntingtin gene and contains a CAG repeat number of around 150. The symptoms in R6/2 mice follow the symptoms in human HD with brain atrophy, movement disorder, weight loss and cardiac dysfunction. The age of onset in the mouse models occurs around 8-9 weeks and death occurs after 12-14 weeks.

Transgenic HD mice of the R6/2 line were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA), the colony was maintained by breeding heterozygous R6/2 males with females from their background strain (F1 of CBA × C57Bl/6). The animals were housed in groups with ad libitum access to chow food and water under standard conditions (12 h light/dark cycle, 22 °C). All animal experimental procedures were approved by the Regional Ethical Committee, Lund, Sweden. The animals were sacrificed at the age of 12 weeks by cervical dislocation and blood samples, and tissue was harvested. Organs were stored frozen at -80 until assay.

### **RNA extraction and real-time PCR**

Brain, liver and kidney from R6/2 (n=10) and wild type (n=10) were homogenized, and RNA was isolated using the QIAzol-reagent (QIAGEN sciences, Germantown, MD USA) according to the manufacturer's instructions. The OD ratio (optical density at 260/280) of RNA was always greater

than 1,8. Reverse Transcription PCR reagents (Fermentas) were used to transcribe mRNA to cDNA. Real time-PCR was then performed using primers for GAPDH, A1M, IDO and TDO obtained from Eurofins MWG Operon. For primer sequences see separate table (Fig 2). The expression was analyzed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA,USA). Raw data were obtained as cycle threshold values (Ct-values), normalized to the Ct-values of mouse GAPDH and expressed as  $\Delta$ Ct (Delta-Ct). Amplification was performed for 40 cycles in iCycler Thermal Cycler (Bio Rad) and data analyzed using iCycler iQ Optical system software.

### **ELISA A1M**

A commercial ELISA kit for detection of mouse A1M was purchased from Usbn Life Science Inc (Wuhan, China) and was used according to instructions from the manufacturer. Serum samples were diluted 500X and incubated for 1h on a 96-well ELISA microtiter plate pre-coated with monoclonal antibody specific for mouse A1M. The plate was washed and incubated with a substrate solution for 25 min. A stop solution was added and absorbance was read immediately at 450 nm. Concentrations were calculated from a standard curve, using Origin 8 software (Microcal, Northampton, MA, USA).

### **ELISA Hb**

A commercial ELISA kit for detection of mouse Hb was purchased from Kamiya Biomedical Company, Seattle, USA. (Cat No KT-399) and was used according to instructions from the manufacturers. Samples were diluted 4000 X as suggested by the kit manufactures, and incubated for 1h on a microtiter plate pre-coated with anti-Hb antibodies. After washing, anti-Hb antibodies conjugated with horse radish peroxidase were added. After a incubation, a second washing and addition of substrate and stop-solution, absorbance was read at 450 nm. Concentrations were calculated from a standard curve as described above.

### **Statistics**

IBM SPSS calculations 20 was used for all statistical calculations. Statistical significance was defined as  $p < 0.05$ .

## **Results**

### **A1M expression in brain, liver and kidney as determined by rtPCR**

Levels of A1M mRNA were normalized to the levels of a house-keeping gene, GAPDH. Mean DCt values of A1M in liver were 21,14 (SD 0,65) for R6/2 mice and 20,89 (SD 0,72) in controls; in kidney 29,31 (SD 1,64) for R6/2 and 28,67(SD 2,59) in controls; in brain 29,55 (SD 2,19) for R6/2 and 29,85(SD 2,48) in controls (Fig 3). There were no statistically significant differences between groups (Mann Whitney U-test).

### **TDO and IDO expression in liver, kidney and brain**

In all PCR experiments samples were measured in triplicates. For a number of individual samples (27/60), liver mRNA levels of TDO in were below detection level (37,84 cycles). However, TDO expression above detection level in all three out of three triplicate samples was seen in 60 percent (6/10) of R6/2 mice as compared to 20 percent (2/10) of wild type controls. Using the Chi-square test statistical significance for the difference was not reached ( $p=0,065$ ).

There was no detectable expression of TDO in kidney in neither R6/2 nor wild type mice (data not shown). We were not able to detect expression of IDO in brain or kidney in any sample from either R6/2 or wt mice.

### **A1M concentrations in serum**

Concentrations of A1M in serum were determined for R6/2 (n=7) and wt (n=5) mice. Mean concentrations were higher in the R6/2 group (9,01 ug/ml, SD 1,71) as compared to wild type (6,68 ug/ml, SD 2,56), but not significantly so ( $p=0,106$  Mann-Whitney U-test) (Fig 4, fig5)

### **Hemoglobin concentrations in serum**

Mean Hb concentrations in serum were significantly higher in the R6/2 mice samples (n=7) as compared to controls (n=5). ( $p=0,048$  Mann-Whitney U-test). Mean Hb concentration for R6/2 was 490 ug/ml (SD 95) as compared to 353ug/ml (SD 298) for wild type mice (Fig 4, fig 6). As illustrated

in Fig 7, serum concentrations of Hb tended to correlate positively with concentrations of A1M, however not significantly so ( $p=0,55$ , Spearman's Rho).

## Discussion

In this study we have measured a significantly elevated serum concentration of Hb in the HD mouse model as compared to healthy mice, and a tendency towards increased serum levels of A1M in the diseased mice, although the latter did not reach statistical significance.

We also found a potentially interesting difference in expression of the KP rate limiting enzyme TDO between healthy and sick mice. The other rate limiting KP-enzyme, IDO, was not detected.

### A1M

Elevated plasma concentrations of A1M was reported previously in a larger study of HD patients (*Olsson et al 2012*). We found a similar tendency in our serum samples. However, in the first group of mice there was no difference in A1M expression in either brain, liver or kidney. We did not have access to serum samples from the first group, so it is not possible to state for sure that there is a discrepancy between serum levels and mRNA expression. However, if this is the case the question remains as to what causes the increase in A1M in serum – where does it come from? The majority of A1M synthesis takes place in the liver, but local up-regulation is possible in other cell types, including blood cells (*Olsson et al 2007*).

Previous studies of uremic patients have shown that plasma levels of 3-HK are greatly increased in the later stages of kidney failure (*Pawlak et al 2009*), and that 3-HK and its autoxidation products are covalently bound to A1M isolated from the urines of hemodialyzed patients (*Sala et al 2004*). We therefore hypothesized that A1M expression would be up-regulated in R6/2 mice, in response to increased oxidative stress and potentially as a defense against increasing levels of the ROS generator 3-HK. As previously discussed 3-HK levels are elevated in brains of HD-patients and R6/2 mice. Looking at mRNA expression in brain only, mean Ct values were marginally lower in the R6/2 group, but the difference was not statistically significant.

## TDO and IDO

TDO mRNA levels were below the detection limit (appr 37.5 cycles) in several of the mouse livers, and a definite  $\Delta C_t$  value could therefore not be obtained in all samples. However, we were able to detect TDO mRNA in a higher frequency of R6/2 liver as compared to controls ( $p=0,068$   $X^2$ -test). This could suggest an up-regulation of the TDO gene in the R6/2 which is potentially interesting as TDO is up-regulated by cortisol (*Oxenkrug 2011, 1969 Young 1981*). Elevated cortisol levels and HPA-axis hyperactivity is a known feature of HD (*Aziz et al 2009*) and R6/2 mice even display a Cushing-like syndrome with muscular atrophy, reduced bone mineral density, abdominal fat accumulation and insulin resistance. (*Björkvist et al 2006*). Genetic inhibition of TDO lead to decreased levels of 3-HK and less neuronal degeneration in HD model flies (*Campesan 2011*). An up-regulation of the TDO gene may therefore be a downstream effect of increased cortisol levels in HD.

IDO is a well studied enzyme and increased activity has been reported for numerous pathological conditions associated with inflammation. As many recent findings (*Soluet and Cicceti 2011*, for review) indicate an activated immune system in HD, IDO activation resulting from a systemic inflammatory challenge could hypothetically explain increased kyn levels in HD patients. Using two different sets of mouse IDO primers previously described in the literature, we were not able to detect any mRNA expression of this enzyme in brain or kidney. This does not exclude that it could be present at sub-detection levels.

An up-regulation of IDO elsewhere could of course still be possible. It should be noted that IDO, in most tissue, is primarily induced by inflammatory cytokines and not expressed or expressed at very low levels under most healthy conditions (*Kwidzinski and Bechmann 2007, Britan et al 2005*).

IDO catalyzes the conversion of trp to kyn. It has been reported by several authors that the kyn/trp ratio is increased in HD (*Forrest 2012, 2010, Guidetti 2000*) Another condition characterized by increased trp degradation and increased plasma levels of 3-HK is AD, where increased IDO expression has been detected in brain. Previous studies (*Sathyasaikumar, Campesan*) indicate that this is not necessarily the mechanism in HD. Direct intrastriatal injections of trp in R6/2 mice did not lead to an



increased production of kyn in brain as compared to controls. In contrast, direct injections of kyn lead to an increased neosynthesis of 3-HK (*Sathyasaikumar 2011*). This strengthens the idea that neurotoxic trp metabolites in HD are derived from an increased influx of kynurenine from the periphery, and that IDO may not necessarily be up-regulated in HD brain.

### **Serum hemoglobin levels**

A previous study unexpectedly found plasma hemoglobin (Hb) levels to be increased, and also correlate with disease severity in two separate cohorts of HD patients (*Olsson et al 2012*). Hb levels were measured in these patients after the finding that they had increased plasma levels of A1M, cell free Hb being a known inducer of A1M expression (*Åkerström 2007, Olsson 2007*). Interestingly, these results could be replicated in this work examining serum A1M and Hb concentrations in R6/2 and control mice.

Measuring plasma or serum levels of Hb has the inherent problem of sampling-induced hemolysis, complicating the interpretation of the results. Hb-concentrations were high also in wt animals as compared to expected *in vivo* levels. However, sampling procedures were the same for R6/2 and wt animals, suggesting that there is actually an increased level of hemolysis or fragility of red blood cells (RBC:s) associated with the HD phenotype.

Interestingly, RBC susceptibility to hemolysis has also been observed in chronic renal disease (*Tankiewicz et al 2005*). We found one study examining the influence of another KP-metabolite, anthranilic acid (AA), on erythrocyte life span and membrane integrity. The study included 26 patients with CRI, and the findings suggested that AA might damage erythrocyte membranes a cause shortening of RBC life span (*Tankiewicz 2005*).

Mechanistically, there are several possible explanations as to how Hb may be involved in HD pathogenesis. Hemoglobin released from damaged erythrocytes is a major pro-oxidant, generator of free radicals and inflammatory mediator (*Everse and Hsia 1997*). Hb can also induce the expression of IDO in dendritic cells, which was proposed as mechanism explaining high serum levels of kyn/trp in patients suffering from hemolytic anemia (*Ogasawara 2009*). *Ogasawara et al* also proposes that IDO

expression is a general control mechanism regulating inflammation resulting from hem mediated toxicity. Hb may thus be pathogenic in HD by its inherent oxidative toxicity, and/or by up-regulating IDO leading to increased levels of kyn and 3-HK.

## **Conclusions**

The metabolism of tryptophan via the kynurenine pathway and its involvement in pathology is clearly a rapidly expanding field of research. Specifically, 3-HK and its neurotoxic effects has been implied in pathogenesis not only of HD, but also in neurological complications of CRI, Alzheimer's disease, stroke, MS, cerebral malaria and major depression. However, to our knowledge, the relationship between 3-HK and A1M has not been studied, other than the discovery that A1M naturally binds to the neurotoxin. We did not find a significant general up-regulation of A1M in HD model mice, but comparison of serum concentrations still support the idea of a role for A1M in this specific disease. Understanding this in more detail would certainly contribute to the understanding of HD, but could also have implications for the understanding of several other conditions.

Abbreviations: 3-HK=3-Hydroxykynurenine A1M=alpha-1-microglobulin, A=Anthranilic Acid, AD = Alzheimer's Disease, CRI=Chronic Renal Insufficiency, Hb=hemoglobin, HD=Huntington's Disease, Htt = Huntingtin, IDO=Indoleamine 2,3 Dioxygenase, ROS=Reactive Oxygen Species KMO=Kynurenine Monooxidase, KP=Kynurenine Pathway, kyn=Kynurenine, TDO=Tryptophan 2,3-dioxygenase, trp=tryptophan

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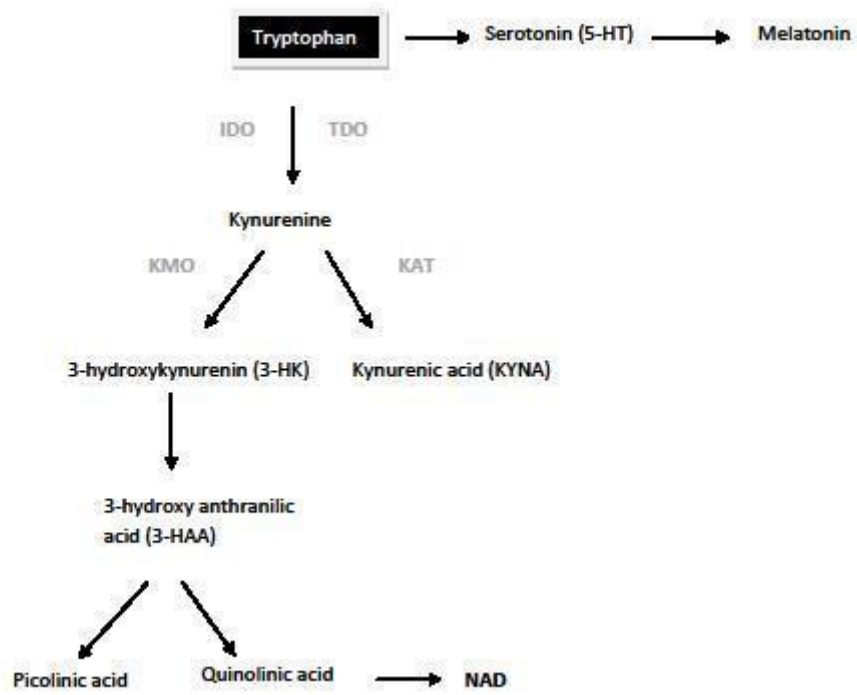
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## Figure legends

1. Overview of tryptophan metabolism via the kynurenine pathway. Major enzymes in grey.
2. Primer sequences (m=mouse, r=rat).
3. Real-time PCR Ct-values of A1M mRNA, normalized to the expression of GAPDH.
4. Mean serum concentrations of Hb and A1M for R6/2 mice and healthy, wild type controls (wt), as measured by ELISA.
5. Mean serum concentrations of A1M in R6/2 mice and healthy, wild type controls (wt). There was a statistically significant difference in mean serum Hb concentrations between the two groups. ( $p=0,048$ , Mann-Whitney U-test).
6. Mean serum concentrations of Hb in R6/2 mice and healthy, wild type controls (wt). Statistic significance was not reached for the difference of mean values between the two groups. ( $p=0,106$  Mann-Whitney U-test).
7. Mean serum A1M concentrations plotted against serum concentrations of Hb. Values for wt ( $n=5$ , white dots) and R6/2 ( $n=7$ , black dots) mice are included. Linear regression coefficients were  $R=0,49$   $R^2=0,24$ . Statistic testing using the Spearman Rho'-test did not confirm positive correlation ( $p=055$ ).



mIDO	Fw 5'-GTG GCC AAG TGG GGG TCA GTG G-3'
mIDO	Rev 5'-CCC TGA TAG AAG TGG AGC TTG CTA C-3'
mIDO2	Fw 5'-CAC TGA GCA CGG ACG GAC TGA GA-3'
mIDO2	Rev 5'-TCC AAT GCT TTC AGG TCT TGA ACG C-3'
mTDO	Fw 5'-TGG GAA CTA GAT TCT GTT CG-3'
mTDO	Rev 5'-TCG CTG CTG AAG TAA GAG CT-3'
mGAPDH	Fw 5'-GAA GAC TGT GGA TGG CCC CTC-3'
mGAPDH	Rev 5'-GTT GAG GGC AAT GCC AGC CCC-3'
rA1M	Fw 5' TTC TTG TTG CTG ACT GCC TGC C -3'
rA1M	Rev 5' TTC TTA ATC CGC CTCA GCC ACG -3'

Figure 2.

<b>Δ Ct values A1M mRNA</b>				
<b>Mouse type/ organ</b>		<b>Brain</b>	<b>Liver</b>	<b>Kidney<sup>2</sup></b>
<b>R6/2</b>	Mean	29,5540	21,1370	29,3125
	Std. Deviation	2,19276	,65451	1,63742
	SEM	0,69341	0,20697	0,81871
<b>wt</b>	Mean	29,8530	20,8870	28,6750
	Std. Deviation	2,48422	0,71650	2,59444
	SEM	0,78558	0,22658	1,05917
<b>Both groups</b>	Mean	29,7035	21,0120	28,9300
	Std. Deviation	2,28568	0,68011	2,17752
	SEM	0,51109	0,15208	0,68859

Figure 3.

<b>Mean serum concentrations of A1M and HB</b>			
<b>Mouse type/ concentration</b>		<b>A1M (ug/ml)</b>	<b>Hb (ug/ml)</b>
<b>R6/2</b>	Mean	9,0143	490,3429
	N	7	7
	SD	1,71214	322,98470
<b>wt</b>	Mean	6,6800	161,0400
	N	5	5
	SD	2,55871	95,15612

Figure 4.

<sup>2</sup> R6/2 n=4, wt=6



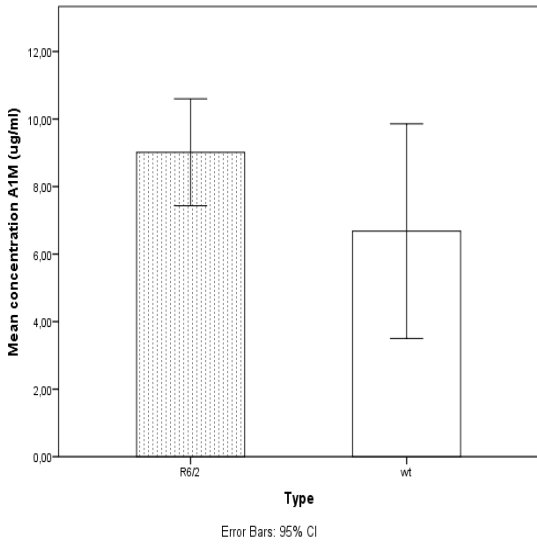


Figure 5.

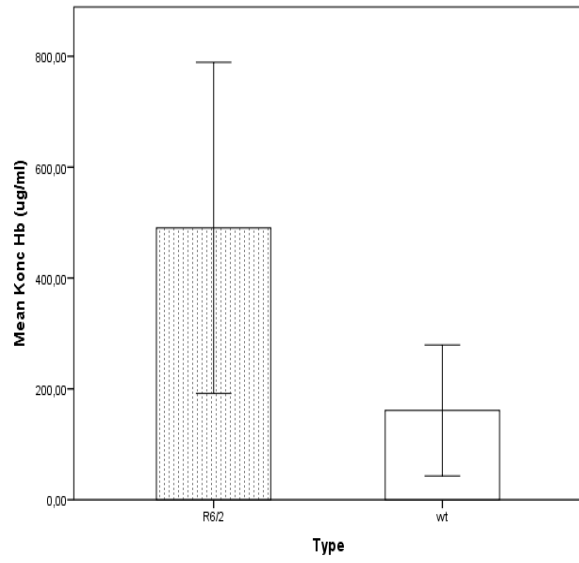


Figure 6.

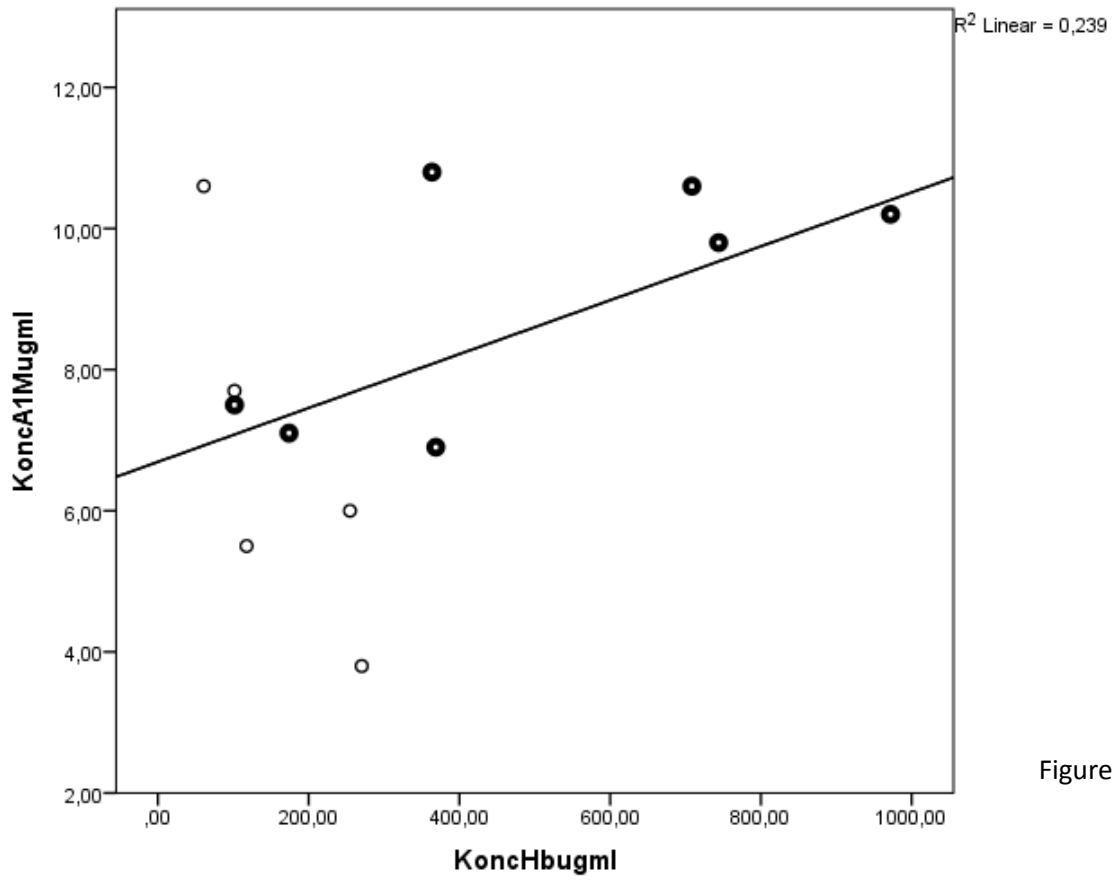


Figure 7.