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Genome-Wide Association Study to Identify Genes Related to Renal Mercury Concentrations in Mice

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Running title: GWAS of renal mercury in mice

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Abstract

Background: Following human mercury (Hg) exposure, the metal accumulates with considerable concentrations in kidney, liver, and brain. Although the toxicokinetics of Hg has been studied extensively, factors responsible for inter-individual variation in humans are largely unknown. Differences in accumulation of renal Hg between inbred mouse strains suggest a genetic inter-strain variation regulating retention or/and excretion of Hg. A.SW, DBA/2 and BALB/C mouse strains accumulate higher amounts of Hg than B10.S.

Objectives: To find candidate genes associated with regulation of renal Hg concentrations.

Methods: A.SW, B10.S and their F1 and F2 offspring were exposed for 6 weeks to 2.0 mg Hg/L drinking water. Genotyping with Microsatellites were conducted on 84 F2 mice for Genome-Wide Scan by using Ion Pair Reverse Phase High Performance Liquid Chromatography (IP RP HPLC). Quantitative trait loci (QTL) were established with R/qtl software. Denaturing HPLC was used to detect single nucleotide polymorphisms for haplotyping and fine mapping in 184 and 32 F2 mice, respectively. Candidate genes (*Pprc1*, *Btrc* and *Nfkb2*) verified by fine mapping and QTL, were further investigated by Real-Time PCR. Genes enhanced by the *Pprc1* (*Nrf1* and *Nrf2*) were included for gene expression analysis.

Results: Renal Hg concentrations differed significantly between A.SW and B10.S mice and between males and females within each strain. QTL analysis showed a peak logarithm of odds ratio score 5.78 on chromosome 19 ($p = 0.002$). Haplotype and fine mapping associated the Hg accumulation to *Pprc1* (Peroxisome Proliferator-Activated Receptor Gamma, Co-activator-Related), encoding PGC-1 protein, a co-activator for proteins involved in detoxification. *Pprc1* and two genes co-activated by *Pprc1* (*Nrf1* and *Nrf2*) had significantly lower gene expression in the A.SW strain, compared to the B10.S strain.

Conclusions: This study supports *Pprc1* as key regulator for renal Hg excretion.

Introduction

Mercury (Hg) is a toxic metallic element which contaminates the environment through anthropogenic as well as non-anthropogenic sources (Tchounwou et al. 2003). The toxicological profile and metabolic fate of Hg for humans and animals depends on form, dose, age and exposure route (Clarkson and Magos 2006; Hultman and Nielsen 2001). Mercury exists mainly in three forms; elemental Hg (Hg^0), inorganic Hg (Hg^{2+}) and organic Hg (methyl- and ethyl- Hg) (Clarkson et al. 2007). The European Scientific Committee on Health and Environmental Risks (SCHER) determined dental amalgams as the dominant source of Hg^0 for the general population by estimating the average daily intake and retention of total Hg and Hg compounds (SCHER 2007). Exposure to methyl Hg (MeHg) from fish consumption has been a concern for decades and risks associated with thimerosal (ethyl Hg) in vaccines are a newer concern (US.EPA 2014). Hg^0 (Halbach et al. 2008) and organic Hg (Clarkson et al. 2007) are transformed into Hg^{2+} in humans and animals in different rates and manners.

The thiol-containing protein glutathione (GSH) binds Hg to form GSH-Hg complexes (Lee et al. 2001; Schlawicke Engstrom et al. 2008; Zalups 2000), and is the primary form in which Hg is transported out of cells (Clarkson et al. 2007). Regulatory pathways of accumulation and excretion are not fully elucidated (Bridges et al. 2014; Zalups 2000). GSH conjugates are transported into proximal tubular cells via organic anion transporters 1 and 3 (Oat1 and Oat3) (Hazelhoff et al. 2012), and are subsequently transported into the urine by multidrug resistance-tolerated proteins (MRPs) (Bridges et al. 2008a, 2011). Polymorphisms in human *ABCC2* gene encoding MRP2 are associated with variations in urinary excretion of Hg^{2+} in populations exposed to Hg^0 vapor from gold mining (Engstrom et al. 2013).

MRPs are regulated by nuclear factor-erythroid 2-related factor 2 (*Nrf2*). *Nrf2* deficient mice exposed to methyl-Hg have increased Hg levels in brain and liver compared to wild type mice (Toyama et al. 2007). GSH is also controlled by the transcription factor nuclear respiratory factor 1 (*Nrf1*) in rats (Yang et al. 2005). Hepatocytes from *Nrf1*- and *Nrf2* knockout mice exhibit lower GSH levels (Chen et al. 2003; Kwong et al. 1999).

Accumulation of renal Hg^{2+} has been reported to vary by sex in humans (Akesson et al. 1991) and rats (Thomas et al. 1987), and between mouse strains (Nielsen and Hultman 2002). In our previous study, between the two mouse strains A.SW and B10.S (Figure 1), A.SW mice accumulated more Hg than B10.S mice. Gender wise, male A.SW showed significantly higher accumulation of Hg than females, while B10.S mice showed the opposite (Ekstrand et al. 2010). Renal Hg measurement data from Ekstrand et al 2010, were used to find candidate genes associated with regulation of renal Hg^{2+} accumulation in mouse. We identified a chromosomal region on chromosome 19 in which the gene *Pprc1* (Peroxisome Proliferator-Activated Receptor Gamma, Co-activator-Related) (PRC, PGC-1), is a potential key regulator in renal Hg accumulation and elimination.

Materials and Methods

Mice

Male and female A.SW (n= 18 and 17, respectively) mice were obtained from Taconic, and B10.S (n= 20 and 23, respectively) mice were obtained from The Jackson Laboratory. F1-hybrids (n=19 males, 20 females) were derived by crossing female A.SW and male B10.S mice. F2-hybrids (n=154 males, 180 females) were obtained by crossing F1-hybrids. Mice were housed at the Animal Facilities, Linköping University, Sweden, kept under controlled environment with 5-10 mice/cage, offered standard mouse pellets (CRME rodent, Special Diets services), and

drinking water *ad libitum*. Studies were approved by the Laboratory Animal Ethics Committee, Linköping, Sweden and all mice were treated humanely with regard to alleviating any suffering.

Exposure and design

All mice (A.SW, B10.S, F1 and F2) were given 2.7 mg HgCl₂/L (Fluka) in drinking water (2.0 mg Hg/L) at age 8-10 weeks, for six weeks before sacrifice (age 14-16 weeks). No mice were exposed during pregnancy. HgCl₂ was mixed with ²⁰³Hg isotope and 1mL drinking water contained 35,000 – 45,000 counts per minute. Radioactivity of left kidney obtained after sacrifice was measured using a gamma counter (Perkin Elmer, 2470 Wizard), and used to quantify renal Hg accumulation.

F2 mice (n= 334) were classified according to renal Hg concentration as “high” (> 5836 ng/g wet weight, the highest concentration in F1 mice), “low” (< 2990 ng/g wet weight, the lowest concentration in F1 mice) and “intermediate” (2990–5836 ng/g wet weight, the range of concentrations observed in F1 mice).

The genome-wide scan was performed on 28 F2 mice selected at random from each group (using the randomized function, RANDBETWEEN, in Microsoft Excel, (McFedries 2010) for a total of 84 mice (44 male and 40 female). Haplotyping was performed on 334 F2 mice, in order to narrow down the Quantitative Trait Loci (QTL) region. Fine gene mapping on 32 F2 mice was performed on a haplotype for detection of candidate genes. Gene expression of candidate genes and genes enhanced by candidate genes was performed on 7 male and 7 female A.SW and B10.S mice (28 mice total), as described in detail below.

SNPs, Microsatellites and Primer design

Single Nucleotide Polymorphisms (SNP) and microsatellites were identified using Ensembl (Flicek et al. 2014) and Mouse Genome Informatics (MGI) data bases (Blake et al. 2014). NCBI/Primer-Blast was used to design primers (Ye et al. 2012). The sex chromosome was excluded from the Genome Wide Association Study (GWAS) because no microsatellites differed between A.SW and B10.S mice (data not shown). Accession numbers were achieved from UniProt database (UniProt 2015).

DNA extraction and genotyping

DNA, isolated from tail, spleen or kidneys was extracted using Wizard, SV Genomic DNA purification System (Promega). These tissues were used to achieve required amount and concentration of DNA. Quantity and purity of DNA was measured with NanoDrop ND-1000 (Thermo Fisher Scientific). Spectrophotometric absorption A₂₆₀/A₂₈₀ was measured 1.8-2.0 and sample diluted to 20 ng/μL. Samples were genotyped using microsatellites (see Supplemental Material Table S1) or designed primers covering SNPs (see Supplemental Material Table S2) (Invitrogen, Life Technologies). PCR conditions were 30 sec at 94°C, 1 min annealing temperature (55-63°C), and 1 min at 72°C for 35 cycles. DNA amplification was verified by gel electrophoresis.

IP RP HPLC and dHPLC

Microsatellites between 2-10bp were detected with Ion pair reverse phase high performance liquid chromatography (IP RP HPLC) on Transgenomic WAVE system (Transgenomic). Mobile phase consisted of Solvent A: 0.1 M triethylammonium acetate (TEAA; Applied Biosystems) and Solvent B: 0.1 M TEAA - 25% acetonitrile (ACN; EM Science). Percentage of B solution, column temperature and flow rate (mL/min) were optimized for each microsatellite.

Detection of SNPs from PCR amplicons were analyzed by denaturing HPLC (dHPLC) using Transgenomic WAVE system. PCR products of F2 mice were pooled with A.SW or B10.S, respectively and denatured by heating at 96°C for 5 min and gradually cool to 25°C for 30 min. PCR products were loaded on DNasep column (Transgenomic) and eluted on a linear ACN gradient in a 0.1 M TEAA buffer (pH 7) with constant flow rate of 0.9 mL/min. Gradient start and endpoint was optimized according to amplicon size. Melting temperature selected for optimal separation of amplified DNA products were calculated by using WAVE maker software, version 3.3.3 (Transgenomic).

Linkage analysis

In order to evaluate candidate genes associated with renal Hg accumulation, linkage analysis was performed. QTL were identified based on LOD (Logarithm of Odds) score profiles derived from a genome-wide single-QTL scan by Haley-Knott regression (Knott and Haley 1992) using the Hidden Markov model (HMM), software R/qtl (version 2.15.3) (Broman et al. 2003). Regression was based on data from 84 F2 offspring for 96 microsatellites covering 19 autosomes with an average spacing of 20 cM (see Supplemental Material, Table S1). Genotype data were 99.7% complete. The genome-wide significance threshold was calculated based on 10,000 permutation replicates. Additional microsatellites were used to narrow down the region with haplotype analysis in which a QTL was found (see Supplemental Material, Table S3). All F2 offspring were genotyped in the QTL region and haplotypes were identified by comparing the genotype of F2 mice with the genotype of A.SW and B10.S. Fine mapping was based on genotyping A.SW, B10.S and F2 mice with SNP markers covering the haplotype, followed by additional QTL on F2 mice.

Sanger sequencing

Sequencing of SNPs in *Lbx1* (P52955) and *Tlx1* (P43345) was performed to clarify if SNPs in background strains A (for A.SW) and C57BL/6 (for B10.S), according to Ensembl- and MGI-databases, are present in A.SW and B10.S, since dHPLC did not show any SNPs. PCR primers covering exons in which SNPs were predicted including exon/intron borders were used to generate PCR products (see Supplemental Material, Table S4). Residual nucleotides were removed by ExoProStar 1-Step (GE Healthcare) and PCR products were sequenced according to a standard protocol for fluorescently labeled dideoxynucleotides (Applied Biosystems, Life Technologies), and separated on a capillary electrophoresis instrument (ABI 3500, Life Technologies).

RNA extraction, cDNA reverse transcription and Real-Time PCR Analysis

Total RNA was extracted from kidneys using RNeasy Mini Kit (Qiagen), following manufacturer's instructions. Quantity and purity were measured with NanoDrop ND-1000 spectrophotometric absorption at A260/A280 value of 1.8-2.0 and diluted to 20 ng/μL. cDNA was synthesized by reverse-transcription of 0.2 μg total RNA using High-capacity cDNA Archive Kit (Applied Bio System). Analysis was performed in duplicates using Applied BioSystems 7500 Fast Real-Time PCR System with Applied BioSystems Taqman Gene Expression Assays (Applied BioSystems). Target gene expression for *Pprc1* (Q6NZN1), *Nrf1*, *Nrf2*, *Btrc* (Q3ULA2) and *Nfkb2* (Q9WTK5) was measured with reporter dye FAM (6-carboxyfluorescein) labeled probes (see Supplemental Material Table, S5). *Pprc1*, *Btrc* and *Nfkb2* were selected since fine mapping and QTL analysis revealed them as candidate genes. *Pprc1* acts as a co-activator for *Nrf1* and *Nrf2*, and was therefore also analyzed (Tsuruoka et al. 2012). Ten genes were evaluated as potential endogenous controls (see Supplemental Material,

Table S6). The criterion for selection of housekeeping genes was based on minimal fluctuation of Ct values between samples in assuming to be independent of Hg exposure. *Gapdh* and *Ppia* were selected as endogenous controls after Ct value determination, by using normfinder (Andersen et al. 2004). Ct variation of *Gapdh* and *Ppia* was below 1 Ct. Geometric mean of *Gapdh* and *Ppia* in each group was used as endogenous controls (see Supplemental Material, Table S7). Results are presented as relative transcription using the comparative Ct method. ΔCt_1 was calculated for each of the target genes in every mouse by subtracting the endogenous control using geometric mean for each sample between *Gapdh* and *Ppia*. ΔCt_2 was calculated by subtracting reference genes in untreated F1 mice (since parental strains are examined). $\Delta \Delta Ct$ was calculated by subtracting ΔCt_2 with ΔCt_1 and relative quantification was finally calculated as $2^{-\Delta \Delta Ct}$.

Statistical analysis

Gene expression and genotype versus phenotype data were tested for normality using D'Agostino-Pearson omnibus normality test, which computes a p-value for the combination of the coefficients of Skewness and Kurtosis (D'Agostino 1986). Data that did not pass the normality test are presented as median \pm interquartile range and comparison between two groups was performed using Mann-Whitney U-test. Data that did pass the normality test are presented as mean \pm SD and comparison between two groups was performed using Welch's T test. Differences with $p < 0.05$ were considered significant.

Results

Characterization of the B10.S and A.SW strains

Genetic Linkage

A highly significant ($p = 0.0002$) QTL, located at 38.46 cM (D19Mit53) on chromosome 19, showed a LOD score of 5.78. QTL's were also detected on chromosome 8 (12.59 cM), 13 (27.48 cM) and 17 (55.48 cM) with LOD scores of ≥ 2 (Figure 2A). Renal Hg accumulation was significantly higher ($p < 0.0001$) in F2 mice that were homozygous for the A.SW allele of D19Mit53 compared with heterozygotes or mice that were homozygous for the B10.S allele, suggesting an autosomal recessive inheritance (Figure 2B).

Haplotype analysis on D19Mit53 (38.46 cM) indicated that 32 of 184 F2 mice were homozygous for the A.SW allele. Additional genotyping with 20 microsatellites (see Supplemental Material, Table S3), spaced between 20.18-56.28 cM, identified a DNA block between microsatellites D19Mit67 (37.98 cM) and D19Mit9 (38.97 cM) in which the 32 F2 homozygous mice were further analyzed with fine mapping. Regression was based on 11 markers consisting of 3 microsatellites and 8 SNPs (Figure 3A), since they were polymorphic between background strains and also between A.SW and B10.S strains. *Lbx1*, *Tlx1* and *Poll* genes within this haplotype are polymorphic between the background strains (Flicek et al. 2014) (Blake et al. 2014), but showed no difference between A.SW and B10.S strains (data not shown), and were therefore excluded. Other genes within this haplotype were not analyzed since they were not polymorphic between the background strains.

Fine mapping narrowed the region to 19:45630547-19:46384795 with a LOD score of 1.94 (Figure 3B). SNP analysis revealed 3 genes to segregate between background strains; *Btrc*,

Pprc1 and *Nfkb2*. *Btrc* displayed 12 SNPs (5'-UTR) and *Nfkb2* 1 SNP (3'-UTR), all located in untranslated regions (UTR) (data not shown). *Pprc1* displayed 7 non-synonymous SNPs denoted SNP¹⁻⁷ that all reside on exon 5 (see Supplemental Material, Table S8).

F2 mice that were homozygous for the A.SW allele (AA) of two SNPs (rs30400427 and rs30815571) in *Pprc1* showed significantly higher Hg accumulation than heterozygous (AB) ($p = 0.0018$) and homozygous B10.S (BB) ($p = 0.0299$) mice (Figure 4). Hg accumulation was not significantly different between *Pprc1* BB and AB F2 mice. Mice that were homozygous for the A.SW alleles of *Btrc* and *Nfkb2* also had higher renal Hg accumulation than heterozygotes (AB) or homozygous (BB) mice (data not shown). Hg accumulation was not significantly different between BB and AB F2 mice on *Btrc* and *Nfkb2* (data not shown).

Comparison of SNP¹⁻⁶ on *Pprc1* between 15 mammals (mouse, rat, human, rabbit, marmoset, vervet-AGM, olive baboon, gorilla, orangutan, cow, sheep, pig, dog, cat and horse) was performed using Ensembl database (see Supplemental Material Figure S1) (Flicek et al. 2014). SNP¹ in mouse were not located in conserved region. SNP² were located in conserved region in 13 mammals and SNP³ was conserved in all 15 mammals. SNP⁴ was in conserved region in 7 mammals. SNP⁵ and SNP⁶ was conserved between mouse and rat only.

Comparison of SNP⁷ (rs30352970) on *Pprc1* between 36 mammals (mouse included) and three species of birds, was performed using Ensembl database (see Supplemental Material, Figure S2) (Flicek et al. 2014). SNP⁷ was conserved in 33 of the mammalian species, and all three bird species. SNP⁷ has the codon GGT (Glycine) in the B10.S strain and AGT (Serine) in the A.SW strain. Conserved region of amino acid sequences was analyzed using multiple sequence alignment software Clustal X (version 2.1), which align sets of amino acid sequences (Larkin et

al. 2007). This was performed on 15 mammalian species (mouse, rat, human, rabbit, marmoset, vervet-AGM, olive baboon, gorilla, orangutan, cow, sheep, pig, dog, cat and horse) which Ensembl database have run a nucleotide alignment against (Supplemental Material, Figure S3). Fourteen of them code for the same amino acid, glycine, as the B10.S strain. Ensembl data indicate that the SNPs in *Btrc* and *Nfkb2* are located on non-conserved regions (Flicek et al. 2014).

Gene expression

Differences in renal mRNA expression of *Pprc1*, *Btrc*, *Nrf1* and *Nrf2* between A.SW and B10.S were examined (Table 1). *Pprc1* acts as a co-activator for *Nrf1* and *Nrf2* (Tsuruoka et al. 2012), and were therefore also analyzed. For males and females combined, *Pprc1*, *Nrf1*, and *Nrf2* mRNA expression was approximately 5 times higher in B10.S compared with A.SW mice (all $p < 0.0001$). Both male and female B10.S mice showed significantly higher expression of *Pprc1* ($p = 0.0014$, $p = 0.0056$), *Nrf1* ($p = 0.015$, $p = 0.0083$) and *Nrf2* ($p = 0.0045$, $p = 0.0049$) compared to male and female A.SW mice. When comparing sexes within each strain, the mRNA expression of *Pprc1* in A.SW male mice was ~ 9 times higher compared to female mice ($p = 0.0103$). *Nrf1* mRNA expression was ~ 12 times higher in male compared to female mice ($p = 0.0001$), and *Nrf2* was ~ 9 times higher in male compared to female mice ($p = 0.0133$). mRNA expression of *Pprc1*, *Nrf1* and *Nrf2* in B10.S mice showed no significant difference between males and females. *Btrc* mRNA expression differed significantly ($p = 0.0437$) between male and female B10.S mice only, whereas *Nfkb2* displayed significantly higher expression between male A.SW and B10.S ($p = 0.0299$).

Discussion

Genome-wide genotyping, haplotype and fine mapping linked renal Hg accumulation to three genes with same LOD scores of 1.94: *Btrc*, *Pprc1* and *Nfkb2*. *Pprc1* gene encodes the protein PRC (PGC-related co-activator), which is a member of the PGC-1 family. Its role is to regulate mitochondrial biogenesis in response to environmental signals (Lin et al. 2005). PRC is a key regulator of both *Nrf1* and *Nrf2* gene expression (Scarpulla 2002; Tsuruoka et al. 2012; Wu et al. 1999). *Nrf1* enhances intracellular GSH-levels that complex with Hg, and *Nrf2* increases MRP levels to stimulate the elimination of Hg-GSH (Bridges et al. 2008b, 2011), via proximal tubular cells into tubular lumen and out of the body (Figure 5) (Clarkson et al. 2007).

We found seven SNPs between A.SW and B10.S strains on *Pprc1* in exon 5 with missense protein variants and amino acid exchanges that may alter gene expression, protein folding, function and regulation (Hamosh et al. 2005). Analysis in Ensembl database showed that SNP⁷ (rs30352970) is conserved in more than 33 mammals (Flicek et al. 2014), which seems to provide a vital function for organisms (see Supplemental Material, Figure S2). A.SW has the codon AGT coding for serine, and B10.S has the codon GGT coding for glycine. When comparing the conserved region of amino acids in 15 mammals, on rs30352970, with multiple sequence alignment with Clustal X (version 2.1), 14 of them have the amino acid (glycine), as the B10.S strain (Larkin et al. 2007) (see Supplemental Material Figure S3). Therefore, the substitution of serine for glycine (Gly1007Ser) may be responsible for higher renal accumulation of Hg in A.SW mice compared with B10.S mice. Ensembl data indicate that none of the SNPs in *Btrc* and *Nfkb2* are in the conserved regions when comparing with a variety of mammalian species (Flicek et al. 2014).

To understand the importance of variants of *Pprc1* on the Hg accumulation, we compared reported renal Hg concentrations in 4 different mouse strains, and nucleotide structure on exon 5 missense variants for *Pprc1*. Significant differences in renal Hg accumulation relative to B10.S mice have been reported for A.SW (Ekstrand et al. 2010; Griem et al. 1997; Nielsen and Hultman 1998), DBA/2 (Griem et al. 1997; Nielsen and Hultman 1998), and BALB/c (Tanaka-Kagawa et al. 1998) strains, which share the same alleles for *Pprc1* SNPs¹⁻⁷ (Table 2), according to Ensembl database (Flicek et al. 2014).

PRC acts as a co-activator on *Nrf1* and *Nrf2* gene expression (Scarpulla 2002; Tsuruoka et al. 2012; Wu et al. 1999). Male and female A.SW mice combined, showed a significantly lower (all $p < 0.0001$) mRNA expression of *Pprc1*, *Nrf1* and *Nrf2* compared to male and female B10.S mice. Polymorphic variants in *Btrc* and *Nfkb2* are localized in the 5'-UTR and 3'-UTR.

Regulatory elements in 5'-UTRs may influence the translation of downstream cistrons (Barrett et al. 2012). We did not find statistically significant differences in *Btrc* or *Nfkb2* mRNA between B10.S and A.SW mice, which suggests that the genetic differences do not influence gene expression. However, since fine mapping and QTL within the haplotype, linked *Pprc1*, *Nfkb2* and *Btrc* as possible candidate genes, SNPs on UTRs of *Nfkb2* and *Btrc* may be in linkage disequilibrium with SNPs on *Pprc1*. This might generate a (high) possibility that PGC-1 gene expression differences between A.SW and B10.S could be due to SNPs in UTRs of *Nfkb2* and *Btrc* instead of non-synonymous SNPs on *Pprc1*.

Male and female B10.S mice had significantly higher expression of *Pprc1*, *Nrf1* and *Nrf2* than male and female A.SW mice, respectively. Our experimental setup mainly concerned strain differences, however, we did observe gender differences in A.SW mice. Expression of *Pprc1*, *Nrf1* and *Nrf2* showed no significant difference between male and female B10.S mice, in contrast

to A.SW mice, which showed a significantly lower expression in A.SW females compared to males. The difference was greater between genders of A.SW strain (up to 12-times) compared to the difference between strains (5-times).

Several factors may explain the gene expression differences between genders and strains; sex hormones, age and duration of Hg exposure. The sex hormone estrogen upregulate transcription of PGC-1 (PRC) in rats. Ovariectomized rats treated with 17β -estradiol subcutaneously showed an increased gene expression of *Pprc1* in cerebral blood vessels compared to ovariectomized rats treated with placebo (Kemper et al. 2014). In our case, female A.SW mice showed a significant decrease of *Pprc1* expression, compared to male. The age of all mice were 8-10 weeks before Hg exposure for 6 weeks, which may have an effect of estradiol levels. *Pprc1* expression showed no significant difference between genders of the B10.S strain which may indicate that the genetic background between A.SW and B10.S differs in the estradiol gene or genes regulating estradiol. As the present study was performed on autosomes only, we cannot rule out the genetic differences on sex chromosomes between strains related to the regulation of gender factors on *Pprc1*. *Pprc1* expression also may vary according to the duration of Hg exposure. *Pprc1* expression was measured only once after 6 weeks of Hg exposure, and it is possible that expression may peak at an earlier or later point in time.

Pprc1 may be a key regulator in the detoxification process of different forms of Hg and also in different organs, since inhaled Hg vapor is oxidized to Hg^{2+} in the blood, and Hg^{2+} can also be formed as part of the metabolism of organic Hg in different rates (Clarkson et al. 2007). GSH-Hg complexes and Nrf's have been identified in liver, kidney and brain (Andersson and Scarpulla 2001) and GSH-Hg complexes appear to be the primary form in which Hg is transported out of cells (Clarkson et al. 2007; Toyama et al. 2007).

Some studies have reported that urinary Hg concentrations in humans correlate with numbers of amalgam surfaces, in which people with more amalgam fillings showed higher urinary concentrations (Barregard 2005; Bates 2006; Dutton et al. 2013). Other studies showed no significant correlation (Ganss et al. 2000; Vamnes et al. 2000). The human *PPRC1* gene has over 120 SNPs in exon 5 (Flicek et al. 2014), and at least one that shares the same position as rs30352970 in mouse is evolutionarily conserved. Genetic variation in *PPRC1* might modify associations between urinary Hg excretion and amalgam fillings in humans (Barregard 2005; Bates 2006; Maserejian et al. 2008).

Conclusion

In conclusion, our findings suggest that *Pprc1* is a plausible candidate for a key regulator of renal Hg concentrations based on a genome-wide scan that linked renal Hg accumulation to chromosome 19 with a LOD score of 5.78, and fine mapping that identified a QTL on *Pprc1* with a LOD score of 1.94. Seven SNPs in *Pprc1* that give rise to different amino acids between A.SW and B10.S mice were also associated with renal Hg concentrations, including two SNPs (SNP² and SNP³) that are in conserved regions in 13 mammalian species, and one (SNP⁷, rs30352970) that is in conserved region in 33 mammalian species and codes for same amino acid in 14 mammalian species. In addition, mouse strains with high renal Hg concentrations share the same nucleotide sequence on *Pprc1*, and significant differences in gene expression between A.SW and B10.S strains were correlated with renal Hg accumulation. Gene expression of *Nrf1* and *Nrf2* in which *Pprc1* regulates, showed the same pattern as the *Pprc1* expression, and are involved in the excretion of Hg.

References

- Akesson I, Schutz A, Attewell R, Skerfving S, Glantz PO. 1991. Status of mercury and selenium in dental personnel: Impact of amalgam work and own fillings. *Archives of environmental health* 46:102-109.
- Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-pcr data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64:5245-5250.
- Andersson U, Scarpulla RC. 2001. Pgc-1-related coactivator, a novel, serum-inducible coactivator of nuclear respiratory factor 1-dependent transcription in mammalian cells. *Molecular and cellular biology* 21:3738-3749.
- Barregard L. 2005. Mercury from dental amalgam: Looking beyond the average. *Occupational and environmental medicine* 62:352-353.
- Bates MN. 2006. Mercury amalgam dental fillings: An epidemiologic assessment. *International journal of hygiene and environmental health* 209:309-316.
- Blake JA, Bult CJ, Eppig JT, Kadin JA, Richardson JE, Mouse Genome Database G. 2014. The mouse genome database: Integration of and access to knowledge about the laboratory mouse. *Nucleic acids research* 42:D810-817.
- Bridges CC, Zalups RK. 2005. Molecular and ionic mimicry and the transport of toxic metals. *Toxicology and applied pharmacology* 204:274-308.
- Bridges CC, Joshee L, Zalups RK. 2008a. Multidrug resistance proteins and the renal elimination of inorganic mercury mediated by 2,3-dimercaptopropane-1-sulfonic acid and meso-2,3-dimercaptosuccinic acid. *The Journal of pharmacology and experimental therapeutics* 324:383-390.
- Bridges CC, Joshee L, Zalups RK. 2008b. Mrp2 and the dmpps- and dmsa-mediated elimination of mercury in tr(-) and control rats exposed to thiol s-conjugates of inorganic mercury. *Toxicological sciences : an official journal of the Society of Toxicology* 105:211-220.
- Bridges CC, Joshee L, Zalups RK. 2011. Mrp2 and the handling of mercuric ions in rats exposed acutely to inorganic and organic species of mercury. *Toxicol Appl Pharmacol* 251:50-58.
- Bridges CC, Joshee L, Zalups RK. 2014. Aging and the disposition and toxicity of mercury in rats. *Experimental gerontology* 53:31-39.

- Broman KW, Wu H, Sen S, Churchill GA. 2003. R/qtl: Qtl mapping in experimental crosses. *Bioinformatics* 19:889-890.
- Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L, et al. 2003. Nrfl is critical for redox balance and survival of liver cells during development. *Molecular and cellular biology* 23:4673-4686.
- Clarkson TW, Magos L. 2006. The toxicology of mercury and its chemical compounds. *Critical reviews in toxicology* 36:609-662.
- Clarkson TW, Vyas JB, Ballatori N. 2007. Mechanisms of mercury disposition in the body. *American journal of industrial medicine* 50:757-764.
- D'Agostino RBS, M. A. 1986. Tests for the normal distribution. In: *Goodness of fit techniques*. New York:Marcel Dekker, 367-420.
- Dutton DJ, Fyie K, Faris P, Brunel L, Emery JH. 2013. The association between amalgam dental surfaces and urinary mercury levels in a sample of Albertans, a prevalence study. *Journal of occupational medicine and toxicology* 8:22.
- Ekstrand J, Nielsen JB, Havarinasab S, Zalups RK, Soderkvist P, Hultman P. 2010. Mercury toxicokinetics--dependency on strain and gender. *Toxicol Appl Pharmacol* 243:283-291.
- Engstrom K, Ameer S, Bernaudat L, Drasch G, Baeuml J, Skerfving S, et al. 2013. Polymorphisms in genes encoding potential mercury transporters and urine mercury concentrations in populations exposed to mercury vapor from gold mining. *Environmental health perspectives* 121:85-91.
- Flicek P, Amode MR, Barrell D, Beal K, Billis K, Brent S, et al. 2014. Ensembl 2014. *Nucleic acids research* 42:D749-755.
- Ganss C, Gottwald B, Traenckner I, Kupfer J, Eis D, Monch J, et al. 2000. Relation between mercury concentrations in saliva, blood, and urine in subjects with amalgam restorations. *Clinical oral investigations* 4:206-211.
- Griem P, Scholz E, Turfeld M, Zander D, Wiesner U, Dunemann L, et al. 1997. Strain differences in tissue concentrations of mercury in inbred mice treated with mercuric chloride. *Toxicology and applied pharmacology* 144:163-170.
- Halbach S, Vogt S, Kohler W, Felgenhauer N, Welzl G, Kremers L, et al. 2008. Blood and urine mercury levels in adult amalgam patients of a randomized controlled trial: Interaction of Hg species in erythrocytes. *Environmental research* 107:69-78.

- Hamosh A, Scott AF, Amberger JS, Bocchini CA, McKusick VA. 2005. Online mendelian inheritance in man (omim), a knowledgebase of human genes and genetic disorders. *Nucleic acids research* 33:D514-517.
- Hazelhoff MH, Bulacio RP, Torres AM. 2012. Gender related differences in kidney injury induced by mercury. *International journal of molecular sciences* 13:10523-10536.
- Hultman P, Nielsen JB. 2001. The effect of dose, gender, and non-h-2 genes in murine mercury-induced autoimmunity. *Journal of autoimmunity* 17:27-37.
- Knott SA, Haley CS. 1992. Maximum likelihood mapping of quantitative trait loci using full-sib families. *Genetics* 132:1211-1222.
- Kwong M, Kan YW, Chan JY. 1999. The cnc basic leucine zipper factor, nrfl, is essential for cell survival in response to oxidative stress-inducing agents. Role for nrfl in gamma-gcs(l) and gss expression in mouse fibroblasts. *The Journal of biological chemistry* 274:37491-37498.
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. 2007. Clustal w and clustal x version 2.0. *Bioinformatics* 23:2947-2948.
- Lee YW, Ha MS, Kim YK. 2001. Role of reactive oxygen species and glutathione in inorganic mercury-induced injury in human glioma cells. *Neurochemical research* 26:1187-1193.
- Lin J, Handschin C, Spiegelman BM. 2005. Metabolic control through the pgc-1 family of transcription coactivators. *Cell metabolism* 1:361-370.
- Maserejian NN, Trachtenberg FL, Assmann SF, Barregard L. 2008. Dental amalgam exposure and urinary mercury levels in children: The new england children's amalgam trial. *Environmental health perspectives* 116:256-262.
- McFedries P. 2010. *Formulas and functions microsoft excel 2010*.
- Nielsen JB, Hultman P. 1998. Strain dependence of steady-state retention and elimination of mercury in mice after prolonged exposure to mercury(ii) chloride. *The Analyst* 123:87-90.
- Nielsen JB, Hultman P. 2002. Mercury-induced autoimmunity in mice. *Environmental health perspectives* 110 Suppl 5:877-881.
- Scarpulla RC. 2002. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochimica et biophysica acta* 1576:1-14.
- SCHER. 2007. *Scientific committee on health and environmental risks preliminary report on the environmental risks and indirect health effects of*

mercury in dental amalgam, approved for public consultation on 29 november 2007.

http://ec.europa.eu/health/ph_risk/committees/04_scher/docs/scher_o_089.pdf.

Schlawicke Engstrom K, Stromberg U, Lundh T, Johansson I, Vessby B, Hallmans G, et al.

2008. Genetic variation in glutathione-related genes and body burden of methylmercury. *Environmental health perspectives* 116:734-739.

Tanaka-Kagawa T, Suzuki M, Naganuma A, Yamanaka N, Imura N. 1998. Strain difference in sensitivity of mice to renal toxicity of inorganic mercury. *The Journal of pharmacology and experimental therapeutics* 285:335-341.

Tchounwou PB, Ayensu WK, Ninashvili N, Sutton D. 2003. Environmental exposure to mercury and its toxicopathologic implications for public health. *Environmental toxicology* 18:149-175.

Thomas DJ, Fisher HL, Sumler MR, Mushak P, Hall LL. 1987. Sexual differences in the excretion of organic and inorganic mercury by methyl mercury-treated rats. *Environmental research* 43:203-216.

Toyama T, Sumi D, Shinkai Y, Yasutake A, Taguchi K, Tong KI, et al. 2007. Cytoprotective role of nrf2/keap1 system in methylmercury toxicity. *Biochemical and biophysical research communications* 363:645-650.

Tsuruoka S, Hiwatashi A, Usui J, Yamagata K. 2012. The mitochondrial sirt1-pgc-1alpha axis in podocyte injury. *Kidney international* 82:735-736.

UniProt. 2015. The uniprot consortium: A hub for protein information. Vol. *Nucleic Acids Res.* 43: D204-D212

US.EPA. 2014. United states environmental protection agency. Available:

<http://www.epa.gov/mercury/thimerosal-vaccines> [accessed 09.25 2014].

Vamnes JS, Eide R, Isrenn R, Hol PJ, Gjerdet NR. 2000. Diagnostic value of a chelating agent in patients with symptoms allegedly caused by amalgam fillings. *Journal of dental research* 79:868-874.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator pgc-1. *Cell* 98:115-124.

Yang H, Magilnick N, Lee C, Kalmaz D, Ou X, Chan JY, et al. 2005. Nrf1 and nrf2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via nf-kappab and ap-1. *Molecular and cellular biology* 25:5933-5946.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-blast: A tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics* 13:134.

Zalups RK. 2000. Molecular interactions with mercury in the kidney. *Pharmacological reviews* 52:113-143.

Table 1. Fold difference in renal mRNA expression (mean \pm SD) following six weeks of Hg²⁺ exposure.

Strain and Gender	Number of mice	<i>Pprc1</i>	<i>Nrf1</i>	<i>Nrf2</i>	<i>Btrc</i>	<i>Nfkb2</i>
A.SW						
Both	14	0,93 \pm 0,71 [#]	0,54 \pm 0,46 [#]	0,71 \pm 0,61 [#]	3,94 \pm 2,68	22,6 \pm 7,97
Male	7	1,37 \pm 0,50 ^{*#}	1,06 \pm 0,14 ^{*#}	1,38 \pm 0,34 ^{*#}	5,01 \pm 3,14	24,53 \pm 8,78 [#]
Female	7	0,15 \pm 0,09 ^{*#}	0,09 \pm 0,04 ^{*#}	0,15 \pm 0,08 ^{*#}	2,88 \pm 1,50	20,68 \pm 6,53
B10.S						
Both	14	4,29 \pm 1,33 [#]	3,04 \pm 1,44 [#]	4,13 \pm 1,52 [#]	6,64 \pm 3,84	14,58 \pm 8,58
Male	7	3,99 \pm 1,52 [#]	2,40 \pm 0,90 [#]	3,43 \pm 1,10 [#]	9,19 \pm 3,53 [*]	10,56 \pm 3,49 [#]
Female	7	4,20 \pm 1,5 [#]	2,70 \pm 1,05 [#]	3,96 \pm 1,33 [#]	4,09 \pm 1,98 [*]	18,61 \pm 10,13

Gene expression in kidney obtained from male and female A.SW and B10.S mice exposed to Hg²⁺ for six weeks. Table is presented as mean \pm SD of fold change in each group. ^{*} Significant difference (P < 0.05) between genders within a strain. [#] Significant difference between strains. (p = 0.0021, Welch's test). *Gapdh* and *Ppia* were used as endogenous controls, fold change is relative to one unexposed F1 mouse (reference sample).

Table 2. *Pprc1* genotypes according to strain

<i>Pprc1</i> SNP	B10.S	A.SW	DBA/2	BALB/c
rs30400427 SNP ¹	A	G	G	G
rs30815571 SNP ²	G	A	A	A
rs30566249 SNP ³	C	T	T	T
rs30507907 SNP ⁴	T	C	C	C
rs30750332 SNP ⁵	A	G	G	G
rs30360955 SNP ⁶	C	T	T	T
rs30352970 SNP ⁷	G	A	A	A

Nucleotide structure on exon 5, missense variants (SNP¹⁻⁷) on *Pprc1* in B10.S, A.SW, DBA/2 and BALB/c strains. SNPs on B10.S and A.SW strains were confirmed with dHPLC. SNPs on DBA/2 and BALB/c strains were confirmed with Ensembl database (Flicek et al. 2014).

Figure Legends

Figure 1. Kidney Hg concentrations. Mercury deposition in mouse kidney of male and female A.SW and B10.S mice, exposed to 2 mg Hg/L drinking water for 6 weeks. Data obtained from previous study (Ekstrand et al. 2010). Figure is presented as mean \pm SD, ** $p = 0.0041$, *** $p < 0.0001$ (Welch's test).

Figure 2. A) QTL on autosomes and B) effect plot. A) Genome-wide scan ($n = 44$ male, 40 female F2) on autosomes was performed to identify QTL associated with Hg accumulation in kidney. LOD score shown on the y-axis indicate a high association with microsatellite D19Mit53 on chromosome 19 with a LOD score of 5.78, *** $p = 0.0002$. **B)** Graph of mean \pm SD renal Hg concentration (ng/g wet weight) according to D19Mit53 genotype. AA = homozygous for the A.SW allele, BB = homozygous for the B10.S allele, AB = heterozygote, **** $p < 0.0001$ (Mann-Whitney test).

Figure 3. A) Fine mapping and B) QTL. A) Markers used for fine mapping in haplotype position between 37.98 and 38.97 cM on chromosome 19, since they are homozygote for the A.SW allele on D19mit53 when analyzing 184 F2 mice. **B)** QTL associated with Hg accumulation in kidney based on fine mapping results on chromosome 19, on 32 F2 offspring homozygote for A.SW on D19Mit53. *Btrc*, *Pprc1* and *Nfkb2* showed a LOD score of 1.94.

Figure 4. SNP genotype in *Pprc1*. Genotype data of F2 offspring on two SNPs, rs30400427 (A.SW: Guanine and B10.S: Adenine) and rs30815571 (A.SW: Adenine and B10.S: Guanine) in *Pprc1*. X-axis demonstrates genotype homozygote for A.SW (AA), B10.S (BB) and heterozygote (AB). Graph is presented as median \pm interquartile range, * $p = 0.0299$, ** $p = 0.0018$ (Mann-Whitney test).

Figure 5. Hg excretion from kidney. Hypothetical conceptual model consistent with our findings. Mercury enters proximal tubular cells via OAT-1, -3 transporter proteins (Bridges and Zalups 2005) already bound to, or binds to sulfhydryl protein GSH, inside tubular cells. MRP1-3 export GSH-Hg complexes out of the cell into tubular lumen and out with the urine (Bridges et al. 2008b; Toyama et al. 2007). *Pprc1* encodes the protein PGC-1 which acts as a co-activator on *Nrf1* and *Nrf2* transcription factors. *Nrf1* regulates production of GSH levels (Chen et al. 2003; Kwong et al. 1999; Yang et al. 2005) and *Nrf2* regulates production of MRP-1, -2 (Toyama et al. 2007).

Figure 1.

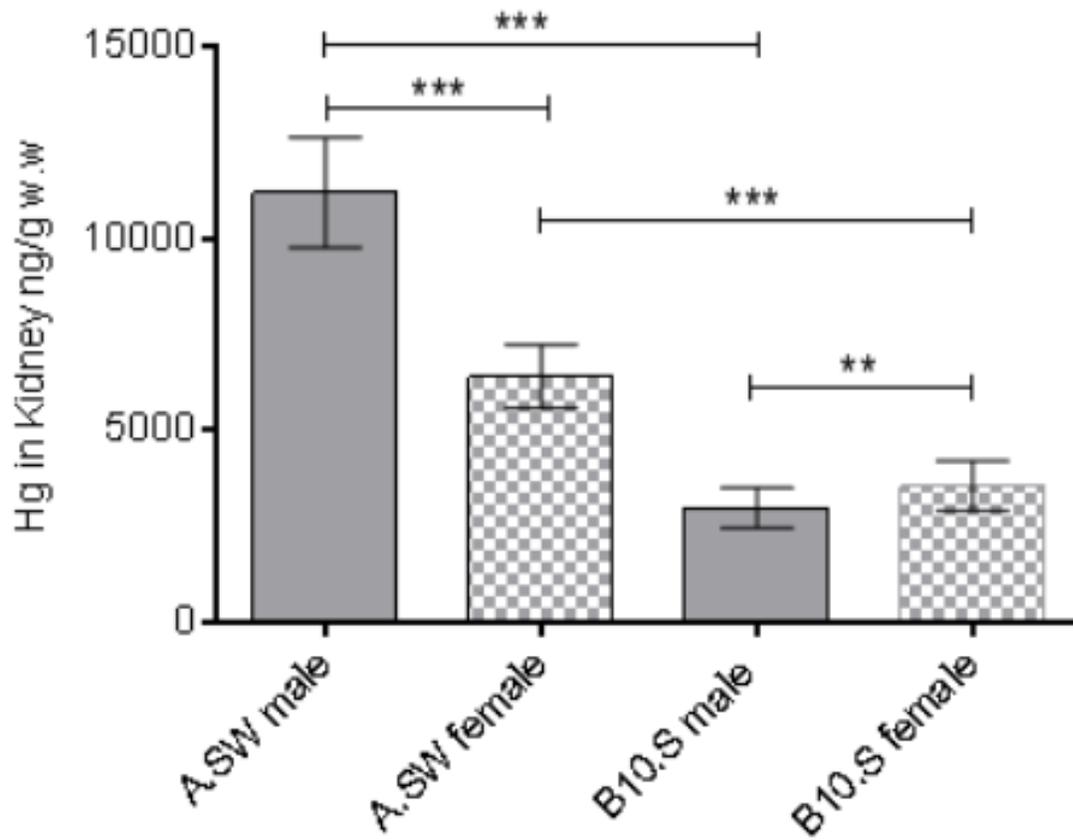
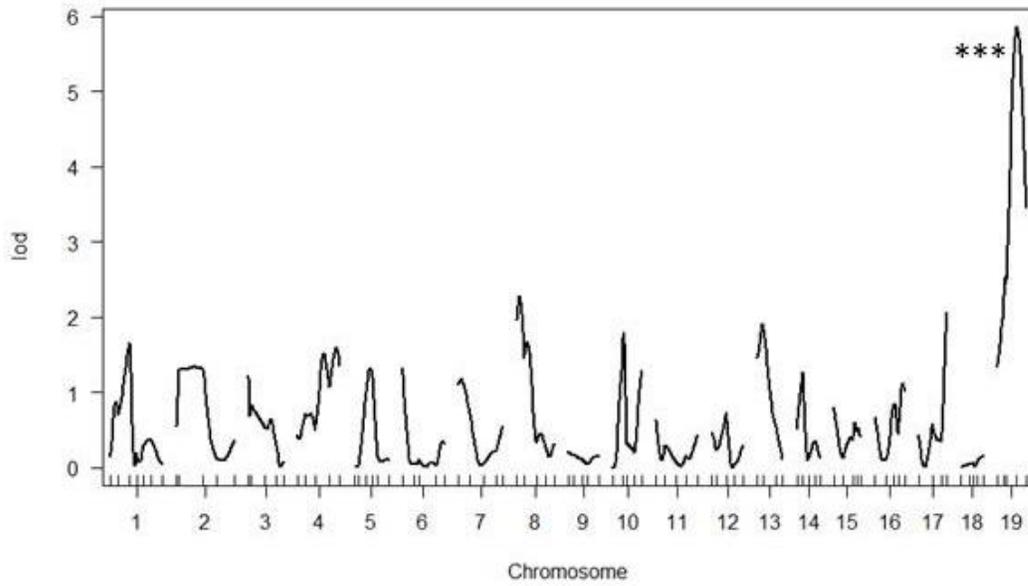


Figure 2.

A



B

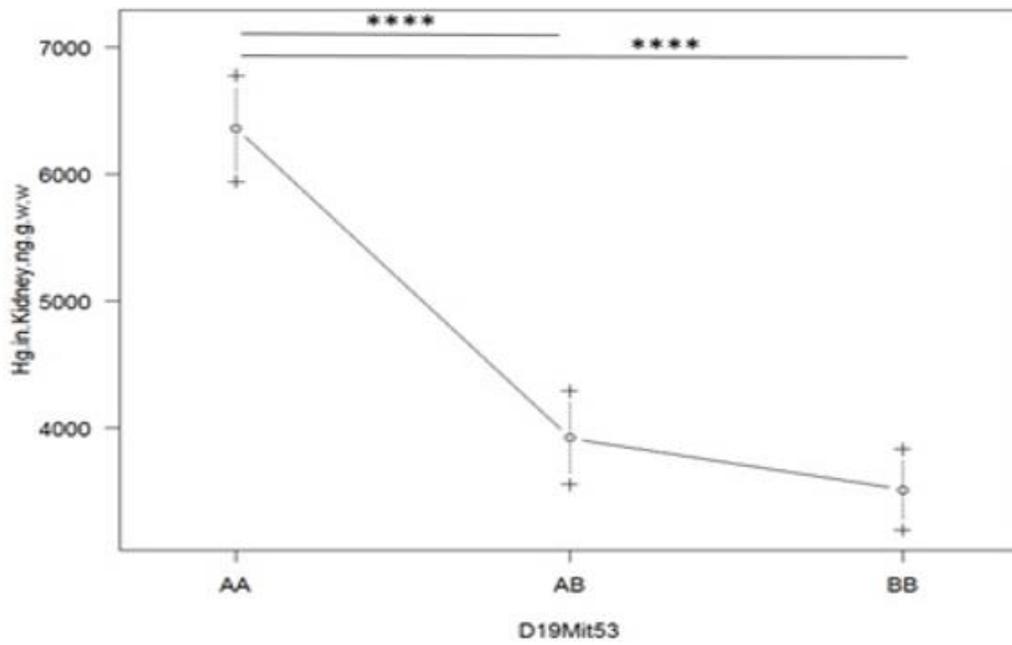


Figure 3.

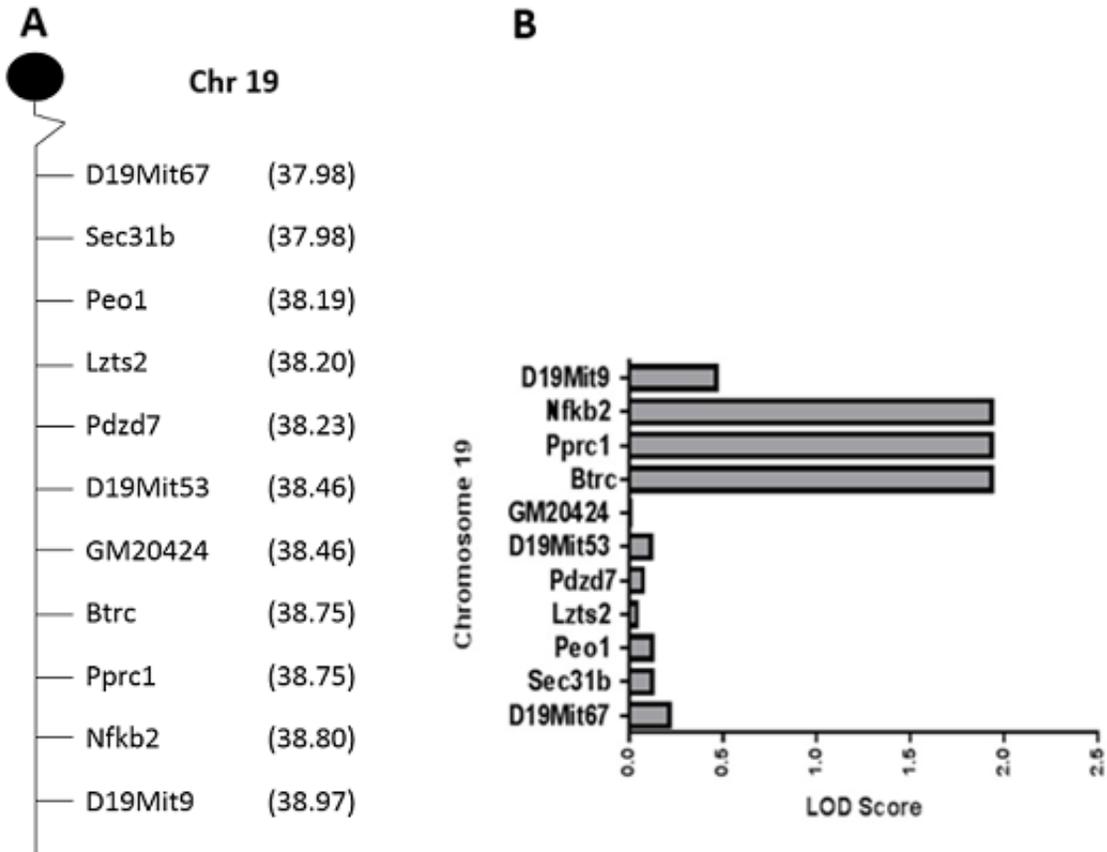


Figure 4.

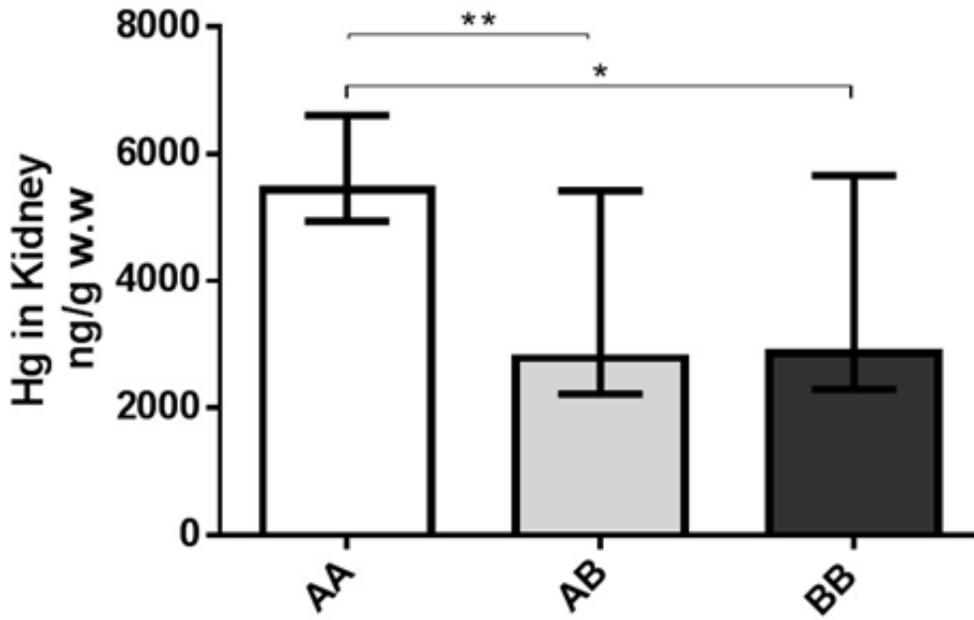


Figure 5.

