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Title page

The ontogeny and population variability of human hepatic NAD(P)H dehydrogenase quinone oxido-reductase 1 (NQO1)

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Abbreviations: AIC, Aikake's informative criteria; BMI, body mass index; BSA, body surface area; CL, clearance; CL_{int}, intrinsic clearance; CPPGL, cytosolic protein per gram of liver; CO, cardiac output, DCPIP, *2,6*-dichloroindophenol; fu_{inc}, fraction unbound; HRP, horse radish

peroxidase; Ki, concentration causing 50% inhibition, K_m, substrate concentration at half maximal velocity*;* NAD(P)H, Nicotinamide adenine dinucleotide phosphate; NQO1, NAD(P)H quinone oxidoreductase; Q, flow; PBST, phosphate buffered saline with tween 20; V_{max}, maximum rate of reaction**.**

Abstract

The NAD(P)H dehydrogenase quinone oxido-reductase 1 (NQO1) enzyme is an antioxidant and metabolic enzyme that performs two electron reduction of quinones and other chemicals. Based on the physiological role(s) of NQO1, we hypothesized that expression and activity of this enzyme would vary with age and other demographic variables. Cytosols from 117 archived human livers were investigated for changes in NQO1 with age, sex, obesity and ethnicity. Protein expression, but not activity, of NQO1 was weakly negatively correlated with age (Spearman r = -0.2, p = 0.03). No sex differences were observed for either protein expression or activity and for ethnicity; Caucasians had greater NQO1 activity than Asians ($p < 0.05$). Overweight children had significantly higher NQO1 activity as compared to ideal weight children ($p < 0.05$), although this difference was not observed in adults. These findings establish that NQO1 is approximately as active in children as adults. However, modeled NQO1 clearance (both allometric and physiologically-based pharmacokinetics), predicted maturation at 23 - 26 years. This is almost certainly an over-estimate, with error in the model resulting from a small sample size and inability to scale for age-related changes in hepatic cellularity and/or cytosolic protein content, and indicates a delay in reaching maximum clearance through the NQO1 pathway that is affected by physiological development as much, or more than, biochemical development. Obesity may increase hepatic NQO1 activity in children, which is likely a protective mechanism in oxidative stress, but may also have significant implications for drug and chemical disposition in obese children.

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Introduction

Throughout the human lifespan, hepatic capacity for drug and chemical disposition can change dynamically. This may be a result of changes in the expression and activity of metabolic and transport proteins, as well as physiological changes that occur in the liver with ageing (Tajiri and Shimizu, 2013; Coughtrie, 2015; Miyagi and Long-Boyle, 2015). The body's capacity for chemical, hormonal and nutrient homeostasis is regulated through a complex series of physiological and biochemical interactions. We are progressively beginning to determine the enzyme expression patterns that are associated with different life-stages, such as how neonates differ from children, adults and the elderly (Tajiri and Shimizu, 2013; Coughtrie, 2015; Miyagi and Long-Boyle, 2015).

The NQO1 enzyme (EC 1.6.5.2) is a cytosolic protein that performs a two-electron reduction converting quinone species to hydroquinones (Dinkova-Kostova and Talalay, 2010). In doing so, NQO1 prevents the one electron reduction producing semi-quinone radicals and therefore mediates a detoxification reaction (Dinkova-Kostova and Talalay, 2010). The crystal structure of NQO1 was elucidated in 2000 and confirmed that the co-factors, NADP and NADPH, share the same binding site as the substrates, suggesting a ping-pong mechanism of catalysis (Faig et al., 2000). The NQO1 enzyme is critical for bioreductive activation of anti-tumor drugs such as Mitomycin C, where higher levels of NQO1 are present in tumors due to hypoxic induction, hence tumors showing reduced levels of NQO1 activity do not respond as well to treatment (Siegel et al., 2012). Moreover NQO1 can reduce toxic metabolites of therapeutic drugs such as the quinoneimine metabolites of diclofenac and mefenamic acid, thereby providing a protective path for detoxication (Vredenburg et al., 2014).

Associations between NQO1 polymorphisms, in particular the low-activity C609T variant, and childhood diseases such as asthma and leukemia have been demonstrated (David et al., 2003;

Guha et al., 2008; Goodrich et al., 2009; Vijayakrishnan and Houlston, 2010; Li and Zhou, 2014). In the adult population, increased cardiovascular and cancer risks (Han et al., 2009; Martin et al., 2009; Kolesar et al., 2011; Lin et al., 2014), as well as increasing severity of Alzheimer's disease in the elderly have also been linked to NQO1 polymorphisms (Tsvetkov et al., 2011)**.** Given these associations with childhood diseases and diseases of ageing, the NQO1 enzyme is under-studied with respect to ontogeny. A single previous study performed in 27 pediatric liver samples demonstrated no differences in NQO1 activity with age, although a weak trend towards increasing activity from birth was observed (Miyagi et al., 2009). In that study, protein levels were not analyzed, and comparisons to adults or the elderly were not performed. Hence, it is unknown whether the susceptibility to disease reported is from constitutively low (polymorphic), developmentally low (pediatric) or declining (elderly) NQO1 activities, or a combination of these factors.

In the present study, we have characterized the expression and activity of NQO1 in the human liver from birth to old age, including pharmacokinetic modeling to predict hepatic clearance maturation through NQO1 pathways. Since NQO1 has been associated with diseases of childhood and of ageing, we hypothesized that the activity of the enzyme would be altered constitutively with the ageing process, providing insight into developmental aspects of enzyme regulation. Additionally, we investigated the impact of covariates sex, ethnicity and obesity and the potential influence these factors may have on NQO1.

Materials and Methods

Sample archive and preparation. An archive of 117 human liver cytosols was collected from commercial (Cellz Direct, Carlsbad, CA, USA; PuraCyp, Carlsbad CA, USA and Xenotech, Lenexa, KS, USA) and Biorepository sources (Hawaii Biorepository, Honolulu, HI, USA and National Disease Research Interchange, Bethesda, MD, USA). This study was approved as exempt by the University of Hawaii Institutional Review Board on Human Ethics.

Commercial samples were kept at -80 °C until use. Tissue samples collected from the biorepositories were processed to prepare cytosolic fractions as follows: tissues were suspended in 0.1 M Tris-HCl with 5 mM $MgCl_2$ and 2 mM phenylmethylsulfonylfluoride and homogenized using a mechanical homogenizer (Tissue Tearor™, Cole-Palmer, Vernon Hills, IL). The total lysate was subsequently centrifuged for 20 min at 10,000 x g and the resulting supernatant centrifuged for 1 h at 100,000 x g to purify the cytosolic fraction. The protein content of all cytosolic fractions were measured using the bicinchoninic acid method and bovine serum albumin as standard (Smith et al., 1985).

Western Blot for NQO1 protein presence. Detection of NQO1 protein by immunoblotting was performed using SDS-PAGE gels (10%) to resolve 10 µg of liver cytosol. Each sample was analyzed on at least two separate gels. The proteins were transferred onto PVDF membranes using a semi-dry system at 12 V for 45 min. Membranes were blocked with 5% nonfat milk powder in PBS with 0.05% Tween-20 (PBST) for 1 h at room temperature. Primary polyclonal rabbit anti-NQO1 (ab34173, Abcam, Cambridge, MA) was added at 1:1000 for 16 h at 4 ^{o}C. Membranes were then washed three times for 10 min in PBST and incubated for one hour with HRP conjugated secondary antibody at 1:5000 (donkey anti-rabbit, Cedarlane, Burlington, ON, Canada) at room temperature. Membranes were then washed three times for 10 min in PBST and finally incubated for one minute in ECL solution before detection on x-ray film.

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A representative pooled liver S9 lysate (10 µg) was included on every blot to normalize the expression across different membranes. Samples were semi-quantified using Image J 1.48v (http://imagej.nih.gov/ij). Briefly, images were scanned as tiff files, opened in Image J and converted to 32-bit grey images. An equal sized square box was drawn and mean grey values were determined for the appropriate band. Background (mean of 3 readings per blot) was subtracted from each band. Liver cytosol expression was normalized to the pooled liver S9 lysate on its own membrane and relative expression to control was reported.

Biochemical determination of NQO1 activity. The method for analyzing NQO1 activity was performed as previously described (Ernster et al., 1972) using 40 µM 2,6-dichloroindophenol (DCPIP, $C_{12}H_7Cl_2NO_2$) as the substrate, with or without the NQO1 inhibitor dicoumarol ($C_{19}H_{12}O_6$, 20 µM final concentration), and the cofactor NAD(P)H at 200 µM final concentration. Protein levels were 0.2 mg in the well. The reaction absorbance was monitored at λ = 600 nm, every 5 seconds for 5 min, and the extinction coefficient ε = 21 $mM^{-1}cm^{-1}$ was used for calculating specific activities. All reactions were monitored for the same length of time (3 min) within the 5 min window, consisting of a linear regression containing 36 data points. During this time less than 10% of substrate was metabolized. Positive controls were a pooled (n = 200) adult human S9 liver samples and showed intra-day coefficient of variation 8.1% and inter-day 10.2%.

Pharmacokinetic modeling and scaling. To evaluate hepatic clearance the enzyme kinetic results were modeled using both the well stirred (1) and the parallel tube (2) equations.

$$CL_{hepatic}\ (\text{L/h}) = \frac{Q_{hepatic} \times f_u \times CL_{int}}{Q_{hepatic} + f_u \times CL_{int}} \qquad (1)$$

$$CL_{hepatic}\ (\text{L/h}) = Q_{hepatic} \times \left(1 - e^{-\left(\frac{CL_{int} \times f_u}{Q_{hepatic}}\right)}\right) \qquad (2)$$

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The intrinsic clearance CL_{int} (V_{max}/K_m) was calculated using experimental V_{max} and the published K_m for DCPIP of 88 µM (Preusch et al., 1991). Hepatic blood flow ($Q_{hepatic}$) of 90 $L \cdot h^{-1}$, an average adult liver size of 1500 g and the cytosol conversion of 80.7 mg/g of liver (Cubitt et al., 2011) were used.

The unbound fraction of DCPIP in the incubation (fu_{inc}) was determined experimentally with equilibrium dialysis. Briefly Micro Float-A-Lyzer dialysis devices (Spectrum Laboratories, Rancho Dominguez, CA, USA) with 10 kD molecular weight cutoff and 100-200 µL capacity were used, as per the manufacturer's instructions. The assay solution (200 µL) consisting of heat-inactivated cytosol, buffer, BSA, DCPIP, and NADH was dialyzed against 2,500 volumes (0.5L) of buffer for 18 hours with continuous stirring. The cytosol used was pooled from 10 individual adults, randomly taken from our archive. The demographics of the pool were: 3 female, 7 male; 8 Caucasian, 1 African American, 1 Hispanic, aged 20-87, mean 56.1 ± 26.4 years of age. Final concentration of protein was 0.2 mg. Three dialysis experiments were performed, with each experiment assayed in triplicate using a spectrophotometer (Molecular Devices, Sunnyvale, CA) at λ = at 600 nm. Comparison was made to the optical density of the same incubation with DCPIP freshly added. The fu_{inc} was defined as optical density DCPIP added to the incubation at time zero minus optical density of solution dialyzed for 18 hours divided by optical density at time zero. The fu_{inc} for DCPIP was 0.595 $\pm$ 0.14 with intra-experiment CV of 8.99% and inter-experimental CV of 23.8%.

To scale for hepatic clearance in children, both allometry and physiologically-based pharmacokinetic (PBPK) analyses were used. The allometric model used to predict $CL_{pediatric}$ was as shown in equation (3).

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$$CL_{pediatric}\ (\text{L/h}) = CL_{hepatic} \times \left(\frac{W_i}{W_{std}}\right)^{\frac{3}{4}} \qquad (3)$$

In equation (3) W_i is the weight of the child and W_{std} is the standard average weight of an adult (70 kg) (EFSA Scientific Committee, 2012).

The activity data were also analyzed using PBPK models derived from SimCYP Pediatric (Certara Inc, St Louis, MO). For pediatric samples, a previously published model (Miyagi and Collier, 2011) was used with exceptions. The present model differs from the published structure due to the inclusion of a pediatric body surface area (BSA) calculation (Eq.4) to account for obesity (Johnson et al., 2005; Johnson et al., 2006). As DCPIP is a weak acid, binding to human serum albumin was assumed, and the unbound fraction for DCPIP was modified by pediatric albumin levels using equation 8 where $[P]_{adult}$ is 44g/L (McNamara and Alcorn, 2002). The CL_{int} was calculated for both pediatric and adult models using equation 9 with the experimental V_{max} divided by K_m and scaled using the cytosolic protein per gram of liver (CPPGL) 80.7 mg/g of liver (Cubitt et al., 2011) and liver volume (Eq.5). The adult cytosolic protein value was used for pediatric samples because a pediatric scalar for CPPGL is not currently available. Pediatric $Q_{hepatic}$ was calculated using equation 6 (Miyagi and Collier, 2011).

$$BSA\ (m^2) = 0.007184 \times Height^{0.725} \times Weight^{0.425} \qquad (4)$$

$$Liver\ Volume\ (L) = \left(Body\ Surface\ Area\right)^{1.176} \times 0.722 \qquad (5)$$

$$Q_{hepatic}\ (\text{L/h}) = 0.265 \times 10^{\left(-0.6492 + 1.943 \times Age - 0.8118 \times Age^2 + 0.08891 \times Age^3\right)} \qquad (6)$$

$$[P]_{Pediatric}\ (\text{g/L}) = 1.1287 \times \text{LN}(Age) + 33.746 \qquad (7)$$

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$$f_{u,pediatric} = \frac{1}{1 + \frac{\left(1 - f_{u,Adult}\right) \times [P]_{pediatric}}{[P]_{Adult} \times f_{u,Adult}}} \quad (8)$$

$$CL_{int}\ (L/h) = \left(\frac{Vmax}{Km}\right) \times CPPGL \times Liver\ Volume \quad (9)$$

Individual adult $Q_{hepatic}$ values for use in the PBPK model were determined using the relationship between cardiac output (CO) and BSA (Dubois and Dubois, 1989; Ghobadi et al., 2011). First, adult BSA (Eq. 4; Dubois and Dubois, 1989) was determined followed by the cardiac index as a function of age in adults (ages > 20) (Eq. 10). The cardiac index was then used to determine CO (Eq. 11) and this value was scaled to 27% for hepatic blood flow and used for adult $Q_{hepatic}$ (instead of the average 90 $L \cdot h^{-1}$) in the PBPK models (Howgate et al., 2006).

$$Cardiac\ index\ (L/min/m^2) = 3 + (-0.01 \times (age\ (yrs)) - 20) \quad (10)$$

$$Cardiac\ output\ (L/h) = cardiac\ index\ (L/min/m^2) \times 60\ (min/L) \times BSA\ (m^2) \quad (11)$$

Statistical analyses and data fitting. Demographic data were not available for all samples; therefore, the figures presented in the results contain exact numbers of samples for each category. Where available, data were analyzed by age, sex, ethnicity and body mass index (BMI, Table 1). The BMI categories were < 18.5 = underweight, 18.5-24.9 = ideal weight, 25-29.9 = overweight and >30 = obese. For pediatric samples, weight was categorized into BMI-for-age percentile using the 2000 CDC BMI-for-age growth charts for children 2-20 years (where it could be calculated) and for children under the age of two, weight-for-age percentiles were

used (http://www.cdc.gov/growthcharts (Kuczmarski et al., 2002). Statistical analyses were performed using GraphPad Prism 5.1 (La Jolla, CA), with $\alpha = 0.05$. All data were tested for normality using the D'Agostino-Pearson omnibus test. Because data were not normally distributed, a Kruskal-Wallis one-way analysis of variance was performed with a Dunn's multiple comparison (for multiple categorized/binned data) or Mann-Whitney U test (binary outcomes). Fitting of the Gaussian curve was performed under medium criteria where iteration occurs up to 1000 times until the fit moves by less than 0.001%. The curve fit was unweighted and unconstrained. For data with continuous variables a line was fitted using linear least-squares regression with 95% confidence intervals (dotted lines). Comparison of model fits for preferred model was with F-tests and Aikake's informative criteria (AIC).

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Results

NQO1 Protein determination. The protein expression of NQO1 was determined by Western blot (Fig. 1A) in 108 samples and resulted in a median of 1.41 mean pixel density normalized to pooled S9 liver sample, and ranged from no detection (n = 2) to 15.9 mean pixel density. When the data was analyzed with age as a continuous variable there was a significant negative correlation between age and protein expression (Spearman r = -0.2, p = 0.03, Fig. 1B). This was primarily driven by the adult population (21-64 years) with a significant negative correlation (Spearman r = -0.34, p = 0.005), while no correlation was observed for children or elderly. These data suggest that age accounts for 4% of the variation in overall data but 12% in the adult age group. When the individuals were grouped into children (≤ 20 years), adults (21-64) and elderly (≥ 65 years), no significant differences were observed between the groups (Fig. 1C). Categorizing "children" as under the age of 21 is in compliance with National Institute of Heath (USA) guidelines, although the World Health Organization (WHO) generally categorizes children as 18 or under. There was only 2 individuals over the age of 18 (aged 19 and 20) and if these two individuals were excluded from analysis outcomes did not change significantly. Therefore, to best fit the widest definition of "pediatric" and increase power, 20 years and under was used throughout. There were also no detectible differences measured between sexes or ethnicities (Fig. 1D, 1E). Significantly lower expression of NQO1 was detected in obese adults compared to ideal weight ($p < 0.05$) or over weight individuals ($p < 0.01$, Fig. 1F). It is not appropriate to include children in the analyses as their categories use percentiles instead of BMI to assign a weight group (see methods). When children were analyzed separately there was no significant differences between groups within the children population, although a trend of higher protein levels in both underweight and overweight children compared with ideal weight was noticed (Fig. 1G).

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NQO1 activity. Analysis of NQO1 activity indicates no significant differences between children (≤ 20 years), adults (21-64) or elderly people (≥ 65, Fig. 2A) with median (range) of 2.95 (0.79-20.51), 4.11 (1.71-19.05) and 3.69 (1.37-13.14) nmol/min/mg protein, respectively. Using age as a continuous variable, there were also no correlations of NQO1 activities with age for children, adults, elderly or the entire population. If all adults (20-89) are included in the analysis, a modest, but significant correlation of declining NQO1 activities with age is observed ($r = -0.29$, $p = 0.01$) and although significant, age only accounts for 8% of the observed variability in this age bracket. Although some evidence is present to suggest that the activity of NQO1 may decline in old age, when a pleateau-to-1-phase decay model (loss of activity at some age) was compared to a horizontal line (no change in NQO1 activity from 13 days to 89 years) for these data, both the F-test ($F = 1.068$) and AIC ($AICc = -3.165$) rejected the decay model in favor of a horizontal line (Fig. 2B). Therefore, we contend that after birth, NQO1 biochemical activities are constant with age throughout life. The amount of NQO1 protein detected by Western blot correlated significantly with NQO1 activity (Spearman $r = 0.47$, $p < 0.0001$, Fig. 2C) and 22 % of the variation in activity is accounted for by variation in NQO1 specific content.

Further study of the distribution frequency of NQO1 activities in this population shows similar skewness in the data towards individuals with higher activities that is apparent in the column graph comparisons in Figures 1 and 2. The curve-fit for the population distribution (Fig. 3) has an amplitude of 21.48 ± 1.438 individuals, which is in very good agreement with the maximum raw frequency of 28 individuals. The curve fit is considered very good-to-excellent with $R^2 = 0.93$, and a runs test indicated that the residuals did not deviate significantly from the model. Moreover, when assessing the goodness of fit, the curve-fit identified the high frequency of the bin 3.0-3.9 nmol/min/mg protein as an outlier to the fitted curve with apparently higher numbers of individuals than ought to occur. This demonstrates that the frequency analysis is sensitive for

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detecting polymorphisms conferring slow metabolizer phenotypes (which should occur in around 12 individuals in this cohort based on published data, see discussion). The model was adjusted to eliminate the effect of the outliers on fit and returned a definite positive skew to an extensive metabolizer phenotype. The mean activity score in the population was 3.48 ± 1.62 nmol/min/mg protein. Using the fitted averages there are 18 individuals with activities > 4 SD of the mean (representing 15% of the population, $p \leq 0.006$). This implies that the positive skew/extensive metabolizer phenotype is real, and occurs at equal or greater frequencies than genetic polymorphisms. We speculate this is due to environmental responsiveness of NQO1 (as discussed below).

Sex was not associated with differences in NQO1 activity in children, adults or elderly nor when all age groups were combined. However with regards to ethnicity the activity of NQO1 was significantly lower in Asian as compared to Caucasian adults ($p < 0.05$, Fig. 4A) and approached significance in Asians as compared to all other ethnic groups. No significant differences between individual ethnic groups were observed for children (Fig. 4B), likely due to lack of power. When children and adults were combined the Kruskal Wallis test returned a result that ethnicity was a significant source of variability ($p = 0.04$), but no individual group was significantly different compared to each other using post hoc analysis (Fig. 4C).

For measures of obesity, underweight was associated with greater NQO1 activities in adults compared to IW, OW and OB categories although this was not significant due to the small sample size (UW n = 3; Fig. 4E). In children, when NQO1 activity was categorized into UW (<5th percentile BMI-for-age), IW (5 to 85th percentile BMI-for-age) or OW (>85th percentile BMI for age), OW children had a significantly higher NQO1 activity than IW children (Mann-Whitney U-test, $p = 0.037$, Fig. 4F). It is not appropriate to combine adult and pediatric datasets for further analysis because the method of calculating obesity status differs between the two groups.

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PK modeling of NQO1. Using allometric modeling and DCPIP as the substrate for NQO1, the maximum mean clearance reached is 4.36 ± 0.37 $L \cdot h^{-1}$, and the predicted age of NQO1 maturity is 23 ± 1.8 years (Fig. 5A). A one-phase exponential growth curve fit better than a sigmoidal curve or straight line, with an $R^2 = 0.116$, absolute sum of squares = 1074 and Sum of Square = 3.06. The well-stirred and parallel tube model produced near identical results only differing after the third decimal place.

The PBPK analysis using the well-stirred model predicted a maximum clearance of 4.055 ± 0.307 $L \cdot h^{-1}$, and the predicted age of NQO1 maturity (90% of adult clearance) at 26.0 years of age (Fig. 5B). A one-phase exponential association curve was used with an $R^2 = 0.296$, absolute sum of squares = 407 and sum of squares = 2.082. While the PBPK parallel tube model predicted a maximum clearance of 4.175 ± 0.322 $L \cdot h^{-1}$, with the predicted age of NQO1 maturity at 24.9 years (one phase exponential association curve with $R^2 = 0.269$, absolute sum of squares = 470 and sum of squares = 2.238). The models tested for maturation were line, sigmoidal curve and one phase exponential association, with one phase exponential association being the best fit according to AIC (well-stirred, exponential compared with line, AICc -16.74, with 99.98% probability it is correct; parallel tube, exponential compared with line, AICc -15 with 99.95% probability). The sigmoidal curve was ambiguous and no comparative statistical data could be generated.

Even though we contend that NQO1 is a class II developmental enzyme biochemically, it would appear that the combination of biochemical and physiological development serves to delay complete maturation of hepatic clearance through the NQO1 pathway.

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Discussion

Data presented herein demonstrate that activity of the enzyme NQO1 does not appear to be developmentally regulated, with levels of hepatic activity as high in neonates and children as they are in adults. Under the classification system originally proposed by Greengard (Greengard, 1969; Greengard, 1977), where Class I is “higher in fetal life”, Class II is “constant throughout life” and Class III is “develops after birth”, we suggest that NQO1 be classified as a Class II ontogenetic enzyme in the human liver. Notably, Olga Greengard primarily described and investigated metabolic enzymes involved in biochemical pathways such as gluconeogenesis and glycolysis with very few studies on xenobiotic-metabolizing enzymes. Her classifications primarily revolved around cross species comparisons (Greengard, 1969; Greengard, 1977). For the highly conserved enzymes of endogenous biochemical metabolism, it is unsurprising that considerable cross-species similarities or identical functions/roles/regulation occur. However, it is recognized that for enzymes considered to be primarily xenobiotic metabolizing, considerable evolutionary divergence has occurred post speciation. This has resulted in, for example, certain cytochromes P450 (CYP) (e.g. CYP3A4, 3A5 and 3A7) being expressed in humans but not other species, such as rodents (Gonzalez and Nebert, 1990). Specifically with respect to NQO1, all vertebrates carry the NQO1 gene and this produces the same, or a strikingly similar, protein with quinone oxido-reductase functions that can be readily up-regulated under conditions of oxidative stress as is constitutively part of the Ah-Receptor battery (Vasiliou et al., 2006)**.** Despite the presence of true NQO1 orthologues across species, and the undoubted identical cross-species effects of hypoxia; species-specific genetic regulation as well as some differences in environmental activation/suppression based on nuclear receptor and signalling factor presence would be expected. Hence the early Greengard classification system with its pan-species applicability in development may be considered too simplistic for xenobiotic metabolising enzymes. With respect to this point, a recent review by Hines (2008) has suggested that the classification system itself (Classes I, II

and III) can also be applied to describe the ontogeny of xenobiotic-metabolizing enzymes in humans. Although cross-species comparisons are almost certainly doubtful, the classification system and its descriptors seem, at least to these authors, to be appropriate as long as the species of interest is clearly noted.

Another interesting finding was that in the adult population there was significantly lower protein levels of NQO1 in obese individuals compared with ideal weight and overweight adults although activity did not differ. There was no difference in protein expression between ideal weight and overweight adults that was somewhat surprising as it suggests only extremes (BMI > 30) inhibit NQO1 expression. Conversely, we detected higher NQO1 activity and protein in overweight children as compared with ideal weight children. Comparing the data between children and adults is not appropriate because overweight and obesity are defined differently in these two populations. A larger data set would be needed in order to determine the mechanism by which higher hepatic NQO1 protein and activity levels occur in overweight children. Such data could be particularly useful to clinicians and researchers focusing on pediatric cancer, non-alcoholic fatty liver disease and oxidative stress. Recent studies have shown a link between obesity and oxidative stress in humans and mice (Cheng et al., 2008; Chartoumpekis et al., 2013; Martinez-Hernandez et al., 2015) and this may be one of the reasons for higher expression of NQO1 in overweight children.

One plausible explanation for the variability in our finding is that NQO1 is a polymorphic enzyme with low-activity variants (NQO1*2 and NQO1*3) that result in a slow metabolizer phenotype. The finding that Asians (here “Asian” represents Japanese, Filipino, Korean and Chinese descent) have significantly lower NQO1 activity than Caucasians likely relates to a high prevalence of low-activity NQO1 polymorphisms in the Asian population. Previous studies have variously demonstrated that Chinese people have up to 29% slow metabolizer profile (Gaedigk

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et al., 1998), while a 24% prevalence of the slow metabolizer phenotype has been reported in a mixed Asian population (Kelsey et al., 1997). Similarly, the prevalence of the slow metabolizer phenotype has been reported at only 5% in Caucasians, thereby supporting the significant activity differences observed with ethnicity (Kelsey et al., 1997; Gaedigk et al., 1998). That we have not genotyped the livers used here for NQO1 polymorphisms is an acknowledged shortcoming of the study. However, as detailed above, we have been able to use mathematical population-based analysis and published genotype/phenotype frequencies to determine the relative contribution of the slow metabolizer phenotype to our findings. Additionally we are able to tease-out an apparent 'extensive metabolizer' subset of individuals that we know cannot be polymorphic and probably represents environmental plasticity in NQO1 in this population. While the effects of NQO1 slow metabolizer polymorphisms/phenotypes have been extensively studied in the context of susceptibility to cancer and effects on drug metabolism demonstrating environmental mechanisms for enzyme plasticity in human tissues is far more rare (David et al., 2003; Guha et al., 2008; Goodrich et al., 2009; Han et al., 2009; Martin et al., 2009; Vijayakrishnan and Houlston, 2010; Kolesar et al., 2011; Tsvetkov et al., 2011; Li and Zhou, 2014; Lin et al., 2014).

Based on the published data (above), the maximum number of slow metabolizers expected in this cohort is 12, comprised of four Caucasians (out of 82), four Asians (out of 12), two Hispanics (out of eight), and one African American (out of 9). Hence, poor metabolizers are unlikely to account for all of the skewness in the data presented. Visually, Fig. 4 shows a significant number of extensive metabolizers far to the right of the frequency curve peak – causing skewness. The number of individuals demonstrating extensive NQO1 activity is almost twice the number of expected (literature) and observed (population fit). Because the extensive metabolizer phenotype did not localize to a single age, ethnicity, sex or weight group, we do not believe that demographic variables and/or underlying genetics are driving the extensive

metabolizer phenotype. Rather, it is postulated that the data presented are reflective of up-regulation of NQO1 in response to environmental stimuli, as has been previously demonstrated with Ah-receptor ligands**,** the antioxidant response element and hypoxia-inducible-1α in several tissue types (Waleh et al., 1998; Collier et al., 2006; Jetten et al., 2014). These data therefore provide interesting insights into gene-environment interactions of hepatic NQO1.

Allometric and PBPK modeling were used to investigate maturity in hepatic clearance via the NQO1 pathway, using DCPIP as a probe substrate. The maximum predicted clearance was not markedly different between well-stirred and parallel tube equations for either allometry or PBPK, but allometric models predict a slightly higher clearance rate of 4.36 $L \cdot h^{-1}$ with both equations as compared to approximately 4.06 $L \cdot h^{-1}$ and 4.18 $L \cdot h^{-1}$ for well stirred and parallel tube, respectively, returned by PBPK modeling. The predicted age of maximum clearance was 23 years for allometry (both equations). In the PBPK simulation, the age of clearance maturation was 26.0 and 24.9 years in the well-stirred and parallel tube equations, respectively. Both models predict clearance maturation at an unrealistically late age, >23 years, which is not supported by the early detection of NQO1 protein and activity demonstrated herein. This result would suggests that physiologically based liver development is a significant contributing factor to hepatic clearance, more than biochemical maturation of NQO1. In the PBPK model the adults were also modeled to take into account body surface area and thus should alleviate differences due to obesity. Hence PBPK is likely more appropriate, and possibly a more accurate prediction tool than simple allometry (R^2 of 0.3 compared to 0.1, respectively), as others have suggested previously (Jones et al., 2006).

Also of note is that the allometric and PBPK models diverged greatest in individuals with high enzymatic activity. This is as expected as the parallel tube model takes into account metabolism of the drug as it passed through a tube, or tissue, and continuously reducing the concentration

while the well stirred is an instantaneous metabolism model. Thus the parallel tube predicted slightly higher clearance rate (4.18 to 4.06 $L \cdot h^{-1}$), which would be expected with higher metabolic turnover.

Although DCPIP is not used clinically, these data have relevance as a proxy measure for the development of the NQO1 metabolic pathway, which may affect the efficacy and toxicity of a number of anti-cancer drugs as well as the function(s) of the innate antioxidant defense network. The specific compound DCPIP has been investigated as an anti-cancer agent with effects in a number of human cancer cell lines including colon cancer (Cabello et al., 2011), breast cancer (Mondalek et al., 2010) and melanoma (Cabello et al., 2009). Additionally, the growth of breast cancer and melanoma xenografts in SCID-mice could be arrested by DCPIP treatment (Cabello et al., 2009; Cabello et al., 2011). In these studies NQO1 was activating the compound and causing cytotoxicity to the tumor. Additionally, the DCPIP analogue EO9, which is also an analogue of Mitomycin C, has come to market for use in bladder cancer with the trade name Apaziquone (Phillips et al., 2013). This demonstrates that the NQO1 pathway is still of interest, although primarily for activating pro-drugs in chemotherapy.

In conclusion, we propose that hepatic NQO1 be classified as a Class II developmental enzyme – stable from neonatal to adult life biochemically. However, in terms of hepatic clearance, which combines physiological and biochemical development, the NQO1 pathway may take longer to approximate adult levels than would be predicted by biochemistry alone. Our other major finding is that the effects of obesity on NQO1 expression and activity in children should be further studied, particularly with respect to fatty liver disease and oxidative stress. It would appear that obesity increases NQO1 protein levels and activities in children but not adults and the mechanisms of these effects on liver function, as well as on drug and chemical metabolism, is deserving of greater study.

Authorship Contributions

Participated in research design: Rougée, Collier

Conducted experiments: Rougée, Berman, Collier

Performed data analysis: Rougée, Riches, Collier

Wrote or contributed to the writing of the manuscript: Rougée, Riches, Berman, Collier

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Footnotes:

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Figure Legends

Fig. 1: NQO1 protein expression compared with age, sex, ethnicity and obesity.

Protein levels were detected by Western blots (**A**) and normalized to a S9 liver pool (n=200) to reduce variability between gels run on different days. Protein expression with respect to: (**B**) age as a continuous variable, dotted line represents the 95% confidence interval; (**C**) binned age categories; (**D**) sex; (**E**) ethnicity; (**F**) weight categories for adults; (**G**) weight categories for children (horizontal lines = median). Spearman's r and p values for correlation are given with (*) representing p value < 0.05, (**) representing p value <0.01. AA = African American and NH = Native Hawaiian. Obesity categories, measured by BMI, where underweight UW = BMI < 18.5, ideal weight IW = BMI 18.5-24.9, overweight OW = BMI 25-29.9 and obese OB = BMI >30. Weight categories for children where underweight UW (< 5^{th} percentile BMI for age; ideal weight IW (6^{th}-85^{th} percentile), overweight OW (>85^{th} percentile).

Fig. 2: NQO1 activity.

The activity of NQO1 was determined by DCPIP metabolism. The activity was compared with (**A**) age when grouped (horizontal lines = median) or (**B**) as a continuous variable. The amount of NQO1 protein detected by Western blot was compared with NQO1 activity (**C**) through Spearman correlation, with (***) representing a p value < 0.0001 and dotted line represents the 95% confidence interval.

Fig. 3: Curve fit of NQO1 activity and population distribution.

The number of individuals within an activity range was calculated to show skewness of data.

Fig. 4: NQO1 activity compared to ethnicity and weight.

The activity of NQO1 was determined by DCPIP metabolism. Activity was compared with: (**A**) ethnicity of adult population; (**B**) ethnicity of pediatric population; (**C**) ethnicity of the whole population; (**D**) weight as a continuous variable for adults and dotted line represents the 95% confidence interval; (**E**) weight categories for adults and (**F**) weight categories for children (horizontal lines = median). Mann-Whitney U-test were used with (*) representing a p value < 0.05. AA = African American; NH = native Hawaiian; underweight (UW), ideal weight (IW) overweight (OW) and obese (OB).

Fig. 5: PK modeling of NQO1 with DCPIP.

Using (A) allometric and (B) PBPK scaling the hepatic clearance was determined for DCPIP. Well-stirred (□, continuous line) and parallel tube (○, dashed line) analysis are shown.

Table 1: Population demographics

	Age **Mean ± SD**	**Ethnicity**		**Gender**		**BMI**	
Population **Age range** **0.04 - 87 yrs**	**N = 117** **41.3 ± 23.8**	**Caucasian** **Asian** **Native Hawaiian** **African-American** **Hispanic** **Other/unknown**	**N = 82** **N = 11** **N = 4** **N = 9** **N = 8** **N = 2**	**Female** **Male**	**N = 40** **N = 77**	**Underweight** **Ideal weight** **Overweight** **Obese** **Morbidly obese** **Unknown/not included**	**N = 7** **N = 41** **N = 27** **N = 23** **N = 7** **N =13**
Pediatrics ≤ 20yrs	N = 29 7.6 ± 7.2	Caucasian Native Hawaiian African-American Hispanic Other/unknown	N = 16 N = 2 N = 6 N = 3 N = 1	Female Male	N = 6 N = 23	Underweight (<5th percentile) Ideal weight (6-84th percentile) Overweight (>85th percentile) Unknown/not included	N = 4 N = 11 N = 10 N = 4

Adult 20 - 64yrs	N = 71 47 ± 11	Caucasian Asian Native Hawaiian African-American Hispanic Other/unknown	N = 50 N = 11 N = 1 N = 3 N = 5 N = 1	Female Male	N = 25 N = 46	Underweight (BMI ≤ 18.5) Ideal weight (18.6 - 24.9) Overweight (25 - 29.9) Obese (30 - 39.9) Morbidly obese (≥ 40.1) Unknown/not included	N = 3 N = 23 N = 14 N = 20 N = 4 N = 7
Geriatrics ≥ 65yrs	N = 17 75 ± 6	Caucasian Native Hawaiian	N = 16 N = 1	Female Male	N = 9 N = 8	Ideal weight (18.6 - 24.9) Overweight (25 - 29.9) Obese (30 - 39.9) Morbidly obese (≥ 40.1) Unknown/not included	N = 7 N = 2 N = 3 N = 3 N = 2

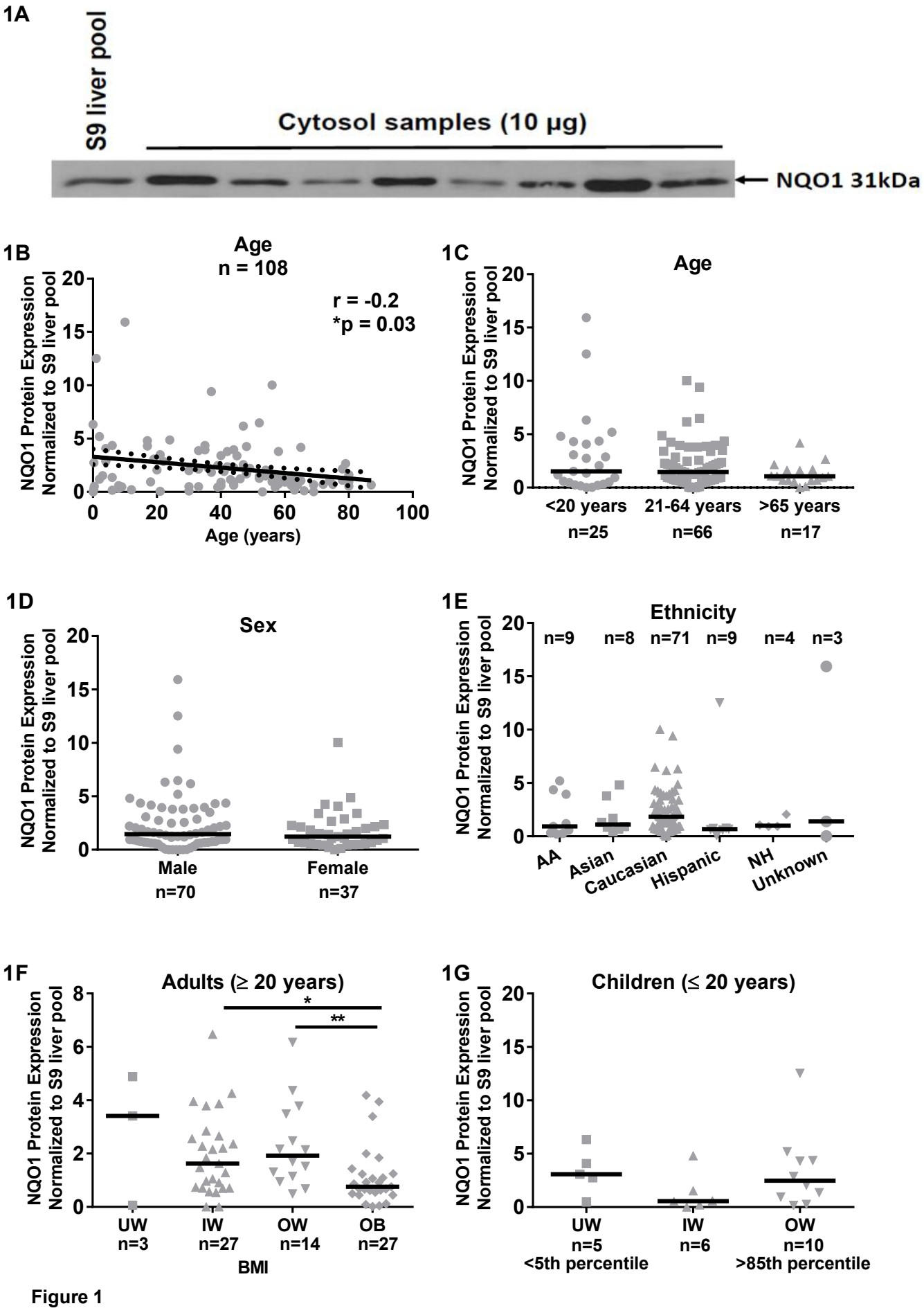
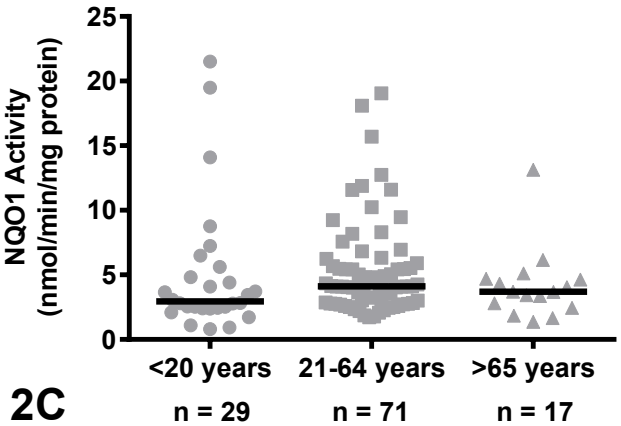
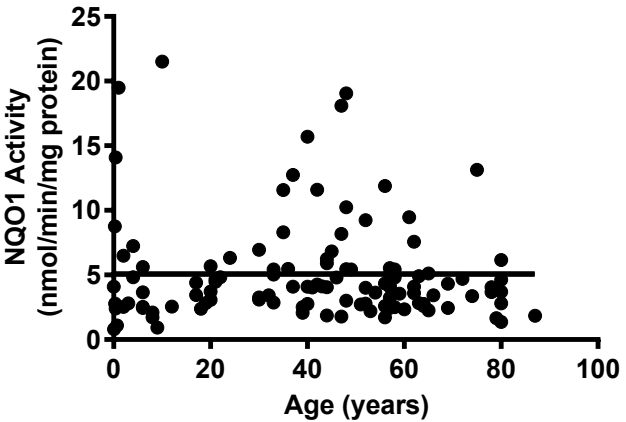

1A
S9 liver pool
Cytosol samples (10 µg)
NQO1 31kDa
1B
Age
n = 108
r = -0.2
*p = 0.03
NQO1 Protein Expression
Normalized to S9 liver pool
Age (years)
1C
Age
<20 years
n=25
21-64 years
n=66
>65 years
n=17
1D
Sex
Male
n=70
Female
n=37
1E
Ethnicity
n=9
n=8
n=71
n=9
n=4
n=3
AA
Asian
Caucasian
Hispanic
NH
Unknown
1F
Adults (≥ 20 years)
UW
n=3
IW
n=27
OW
n=14
OB
n=27
BMI
1G
Children (≤ 20 years)
UW
n=5
<5th percentile
IW
n=6
OW
n=10
>85th percentile

Figure 1


2A
NQO1 Activity
(nmol/min/mg protein)
25
20
15
10
5
0
<20 years
n = 29
21-64 years
n = 71
>65 years
n = 17


2B
NQO1 Activity
(nmol/min/mg protein)
25
20
15
10
5
0
0
20
40
60
80
100
Age (years)

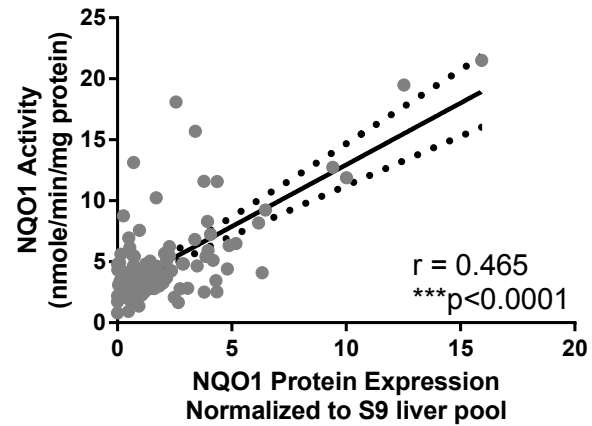

2C
NQO1 Activity
(nmole/min/mg protein)
25
20
15
10
5
0
r = 0.465
***p<0.0001
0
5
10
15
20
NQO1 Protein Expression
Normalized to S9 liver pool

Figure 2

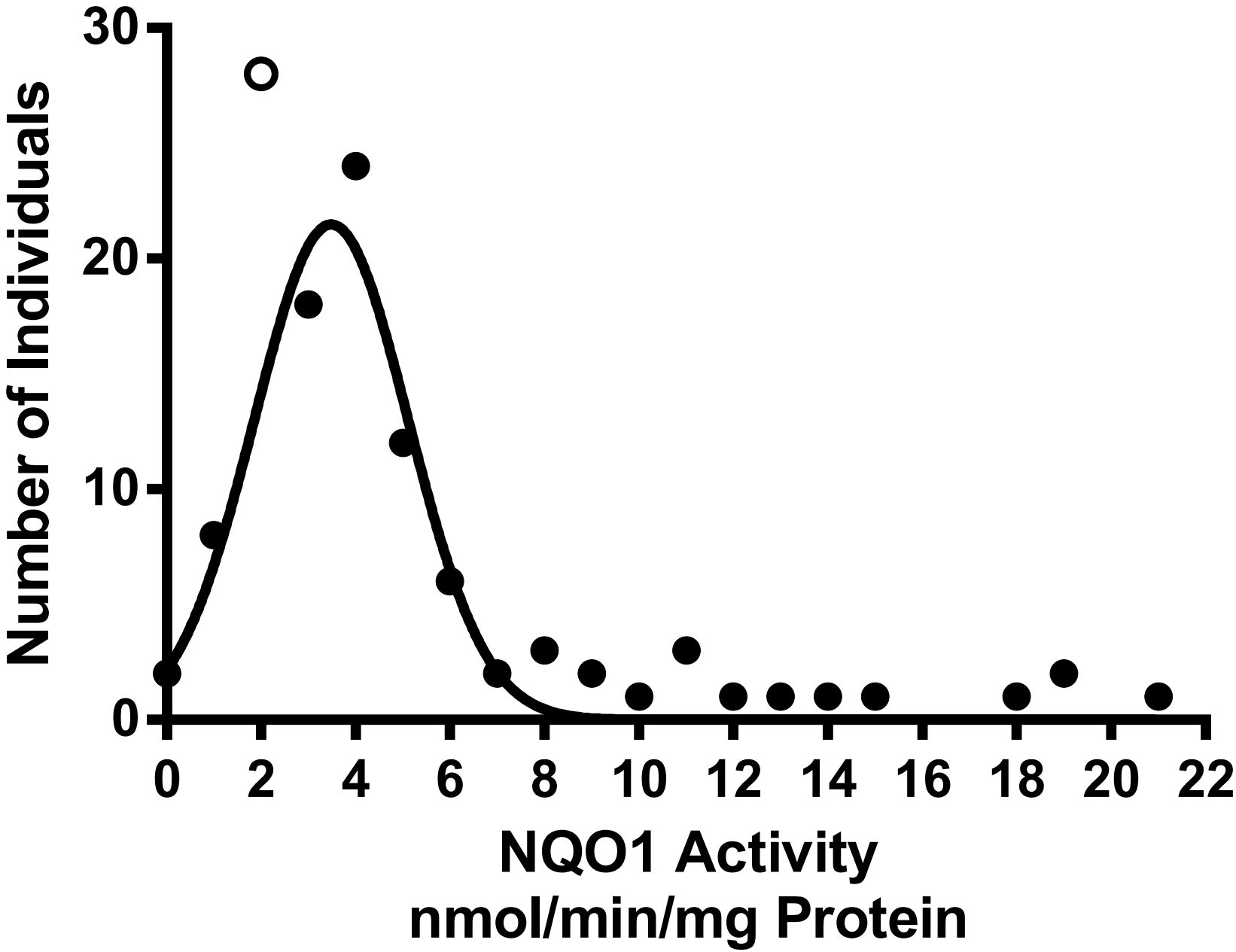

Number of Individuals
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
NQO1 Activity
nmol/min/mg Protein

Figure 3

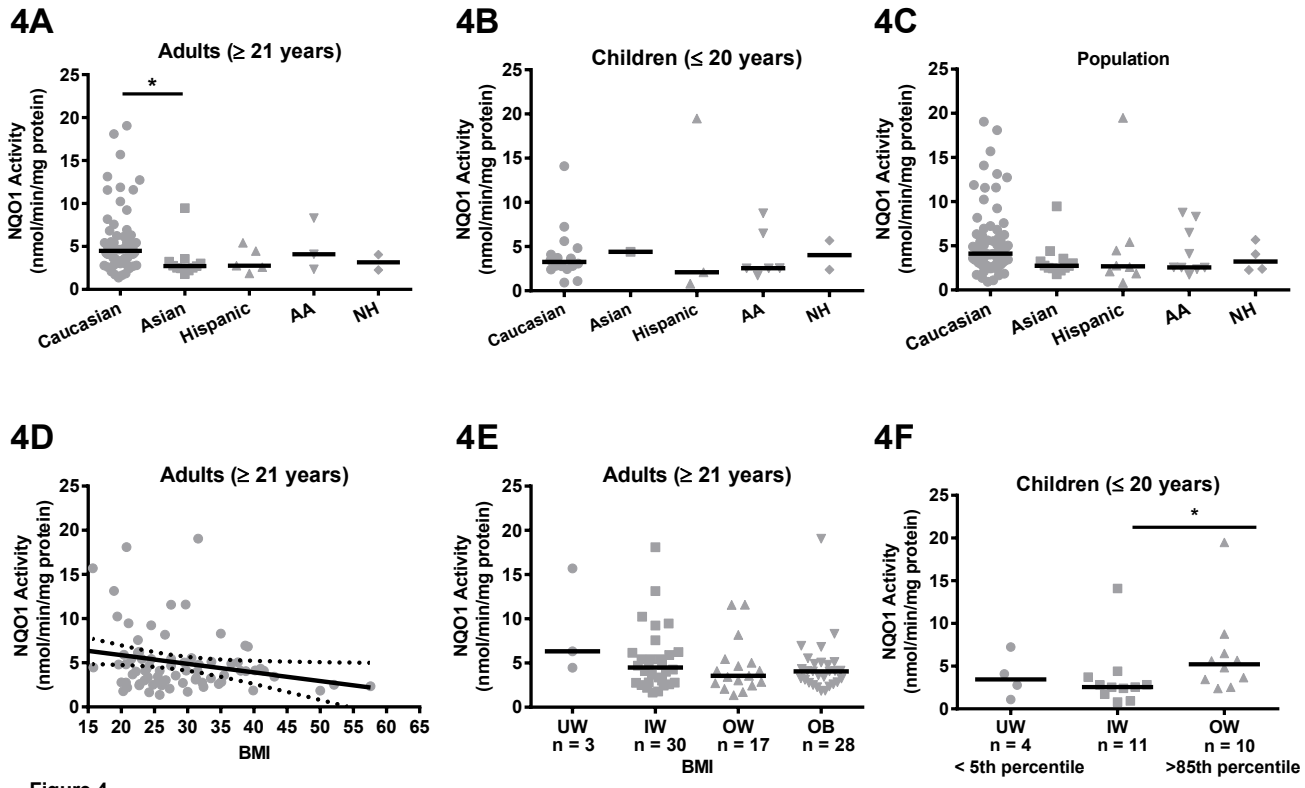

4A
Adults (≥ 21 years)
NQO1 Activity (nmol/min/mg protein)
*
Caucasian
Asian
Hispanic
AA
NH
4B
Children (≤ 20 years)
4C
Population
4D
Adults (≥ 21 years)
BMI
4E
Adults (≥ 21 years)
UW n = 3
IW n = 30
OW n = 17
OB n = 28
4F
Children (≤ 20 years)
UW n = 4
IW n = 11
OW n = 10
< 5th percentile
>85th percentile

Figure 4

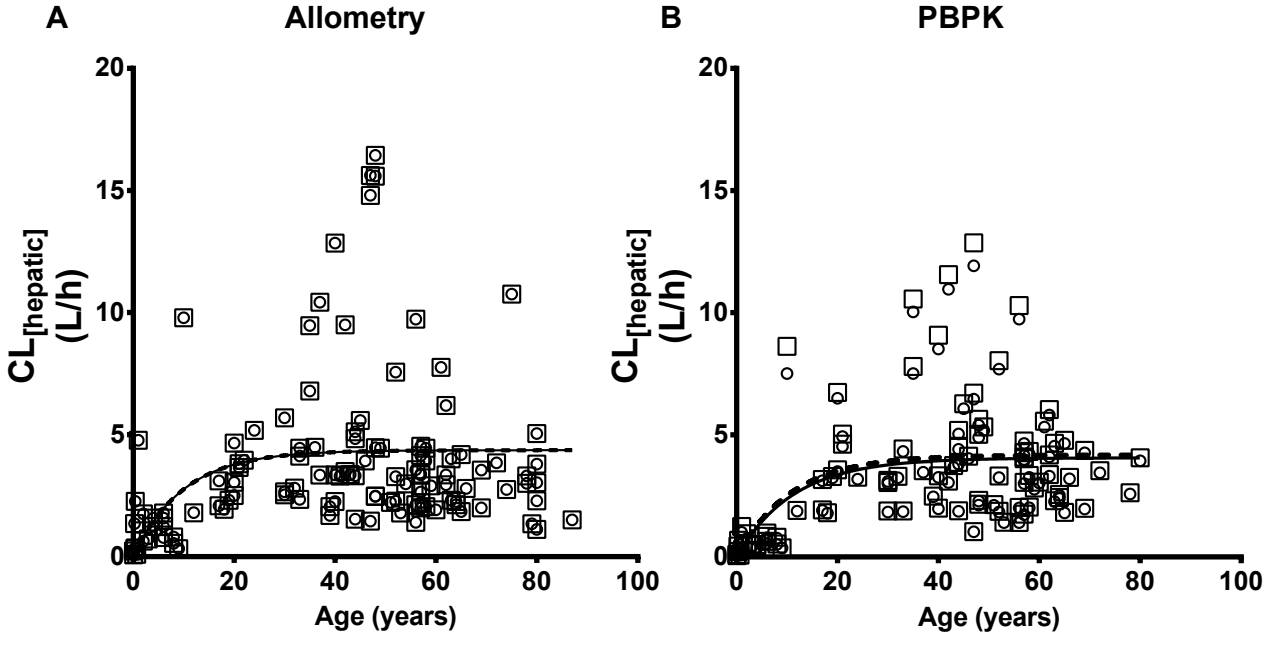

A
Allometry
B
PBPK
CL[hepatic] (L/h)
Age (years)
0
5
10
15
20
0
20
40
60
80
100

Figure 5