



Published in final edited form as:

Clin Chim Acta. 2009 April ; 402(1-2): 67–74. doi:10.1016/j.cca.2008.12.019.

Validation of PON1 enzyme activity assays for longitudinal studies

Karen Huen¹, Rebecca Richter², Clement Furlong², Brenda Eskenazi¹, and Nina Holland¹

¹ Center for Children's Environmental Health, School of Public Health, University of California, Berkeley, CA

² Departments of Genome Sciences and Medicine, Division of Medical Genetics, University of Washington, Seattle, WA

Abstract

Background—Paraoxonase (PON1) enzymatic activity assays are used to characterize sensitivity to organophosphates and oxidative stress. Length of sample storage, temperature and other factors may influence variability of PON1 measurements, especially in longitudinal studies.

Methods—Effects of assay temperature, storage duration up to 7 y (–80°C), freeze-thaw cycles, the type of specimen (serum or heparinized plasma) and assay variability were evaluated for 4 PON1 substrate-specific assays using samples from two pediatric cohorts and laboratory volunteers.

Results—Intra- and inter- assay variation, as well as inter-laboratory variability for PON1 activities were <10%. The effect of storage duration up to 2 y was minimal. However, after 7 y, arylesterase, paraoxonase, and chlorpyrifos-oxonase activities decreased more noticeably. Similarly, while freeze-thaw cycles did not affect the PON1 activities in samples stored < 2 y, this factor was more significant after 7 y for arylesterase. Assay temperature and specimen type also influenced PON1 measurements.

Conclusions—Sources of technical variability of PON1 activity assays, including storage duration, freeze-thaw, and temperature should be monitored and minimized through study design, quality control procedures and statistical methods, especially in longitudinal studies where specimens may be stored for years prior to analysis.

Keywords

paraoxonase; arylesterase; PON1 activity; enzymatic assay; children; storage; technical variability; temperature; organophosphate; pesticides; oxidative stress; freeze-thaw; birth cohort

1. Introduction

Paraoxonase 1 (PON1) is a high-density-lipoprotein (HDL)-associated enzyme, whose multifunctional activities implicate a role in both organophosphate (OP) sensitivity and oxidative stress [1]. Its name stems from the ability to hydrolyze the oxon derivative of parathion, a neurotoxic OP pesticide, and further studies have shown PON1 can hydrolyze additional OPs in vitro, including nerve agents such as sarin and soman [2]. OP pesticides are still widely used in agriculture; for instance, 2 million pounds of chlorpyrifos were applied in the state of California in 2005 [3]. Diseases related to oxidative stress including heart disease,

Address correspondence and reprint request to: Nina Holland, PhD, 50 University Hall, School of Public Health, UC Berkeley, CA 94720-7360, Phone: 510-455-0561, Fax: 510-643-5426, Email: E-mail: ninah@berkeley.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

are some of the leading causes of mortality and morbidity in the U.S. [4]. Attention towards a potential link between cardiovascular disease and PON1 originated from its physical association with HDL. *In vitro* and *in vivo* studies suggest that PON1 has antioxidant properties as it prevents LDL and HDL oxidation [5] and protects against atherosclerosis [6,7]. Understanding the determinants of PON1 variability and how they confer susceptibility to disease or exposures may have broad public health significance.

Experiments in developing animals suggest OP exposure, especially in the young, may influence neurodevelopment and growth [8]. In our longitudinal birth cohort, CHAMACOS, we previously reported an association between *in utero* OP exposure, as measured by urinary metabolite levels, and decreases in gestational duration [9], abnormal reflexes in neonates [10], and mental development at age two, and increases in maternal report of pervasive development disorder. Since PON1 can hydrolyze OP oxon derivatives, it may mediate susceptibility to OP exposures. Berkowitz et al. [11] found that for pregnant women with chlorpyrifos levels above the limit of detection, those women with higher PON1 levels had children with larger infant head circumference at birth. PON1 genotypes, including the polymorphism at position 192, have been associated with preterm birth [12,13]. PON1 genotypes have also been associated with a myriad of other health outcomes related to OP sensitivity and oxidative stress including Alzheimer's disease [14], Parkinson's disease [15], and cardiovascular disease [16]. However, results have been inconsistent. PON1 enzyme levels can range widely even between individuals with the same PON1 genotypes. PON1 status which considers both PON1 genotypes and PON1 levels is a more informative biomarker for use in epidemiological studies than PON1 genotype alone [2,17–19]. Therefore, PON1 studies should utilize PON1 enzymatic assays for determination of PON1 phenotypes whenever possible.

Assays employing spectrophotometric methods to measure the rates of hydrolysis of specific PON1 substrates (i.e. phenyl acetate and paraoxon) have been in use for many years [20,21]. The development of a microplate-based method has allowed for high-throughput measurement of PON1 activity [19,22,23]. As a result, PON1 activity measures have become a viable biomarker of PON1 phenotypic variation in epidemiological studies [2,24]. Previous studies have established experimental conditions for PON1 substrate-specific assays by determining the appropriate substrate, salt, and plasma concentrations [23]. However, few studies have used these assays to compare PON1 phenotypes in the same children at multiple time points [25]. In large longitudinal studies and pediatric disease studies, thousands of samples may be collected at various times over the course of many years. The effects of additional experimental factors such as sample handling and storage become particularly important to address in these studies as they may influence measurements of PON1 levels and enzyme activities and overall interpretation of results.

Several factors can affect biomarker variability. For instance, biologic factors are related to host variability, while technical variation in the laboratory may be due to methodological differences, human error, or other factors [26]. In order to accurately shed light on biologic factors related to biomarker variability, one must minimize the possible sources of technical variation that may affect analyses. For instance, enzymatic assays are generally affected by temperature; ambient temperature can fluctuate day to day, which may influence the measurement of PON1 phenotypes. In large birth cohort studies, variation in specimen handling and storage including length of storage duration at different temperatures (often -20°C or -80°C) and number of freeze-thaw cycles may serve as additional sources of technical variation [27]. Previous studies of PON1 activity have shown little change over the course of 6 months for specimens stored at -80°C [28], however sample storage may span several years in longitudinal studies. Another paper indicated a decrease in paraoxonase activity with increased storage time, but the authors stored samples at -20°C rather than at -80°C [29].

2. Materials and methods

2.1 Study subjects

Samples from 2 ongoing projects were used for this validation study including (1) a pediatric inflammatory bowel disease (IBD) case-control study and (2) a longitudinal birth cohort study, the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS). The IBD cohort was established as part of a pilot study of biomarkers of genetic damage in pediatric patients [30]. Cases and controls matched by age, sex, and race were recruited from hospitals that were members of the Pediatric IBD Consortium [30,31]. The majority of children were Caucasian (82%) and ages ranged from 5- to 17- y (mean = 12 y). CHAMACOS is a cohort study of the effects of pesticide and other environmental exposures on neurodevelopment, growth, and respiratory disease in children from primarily Latino farmworker families in the Salinas Valley, CA [32]. Located in Monterey County, the Salinas Valley is an area of heavy agricultural production where approximately half a million pounds of OPs are applied annually [3]. Human subjects approval was obtained for both projects.

To confirm the overall reproducibility of the PON1 enzyme activity assays between laboratories, a set of CHAMACOS samples (n=22) were run in parallel both at the Holland laboratory at the University of California, Berkeley (UCB), and at the Furlong laboratory at the University of Washington, Seattle (UW), using identical reagents, software, and protocols.

For comparison of PON1 enzyme activity in serum and heparinized plasma, samples from a random selection of children from the IBD cohort (n=38) were used. PON1 enzyme activity assays were performed in parallel samples of each type for each individual.

Inter- and intra-assay CVs of PON1 activity (4 substrate-specific assays) were examined by analyzing multiple aliquots of heparinized plasma from laboratory volunteer samples. Aliquots of these samples were assayed in triplicate in the same experiment and also as many as 20 times in separate experiments. Inter-assay variability was measured using parallel aliquots of the same sample run on different days and within-assay variability was measured by using parallel aliquots of the same sample run on different plates but on the same day. Additionally, these laboratory volunteer samples were used to examine short term effects (less than two years) of assay temperature, storage duration, and freeze-thaw cycles.

To compare effects of storage duration in samples stored > 2 y, a set of randomly selected heparinized plasma samples (n=95) from pregnant women in the CHAMACOS cohort [32] were assayed for PON1 activity after 2 and 7 y of storage at -80°C. These data were compared to PON1 enzymatic assay measurements from another set of CHAMACOS mothers (n=27) whose samples were also assayed after 2 and 7 y of storage. In this set, samples had undergone an additional freeze-thaw cycle between the second and seventh year of storage. These samples were used to determine the effect of freeze-thaw cycles on the PON1 enzyme activity in samples stored > 2 y.

2.2 Blood collection

Blood was collected via venipuncture with and without heparin. Blood samples were separated into various components and then aliquotted, barcoded for identification, and stored (-80°C) at UCB for future use according to Best Practices for Biorepositories [33].

2.3 Determination of PON1 levels and activities

Several substrate-specific kinetic enzyme assays have been developed to measure PON1 phenotype using spectrophotometric methods. The paraoxonase (POase) assay measures the rate of hydrolysis of paraoxon, the oxon derivative of the OP pesticide parathion. Similar assays

(DZOase and CPOase) have been developed for diazoxon and chlorpyrifos-oxon, the derivatives of OP pesticides diazinon and chlorpyrifos. PON1 status determination can be resolved using a two substrate assay, which plots DZOase versus POase activities and reveals a trimodal distribution that resolves subjects by PON1 192 genotype [19]. It should be noted however that the variation measured by the DZOase assay as employed in PON1 status determination is not biologically relevant. The high salt concentration utilized in the assay inhibits the activity of the *PON1*_{192R} alloform even though the *PON1*_{192Q} and R alloforms have equivalent catalytic efficiencies for DZO *in vivo* [24], and is used intentionally to provide better resolution in the two substrate plots. PON1 hydrolysis of the aryl ester, phenyl acetate, is used by researchers to determine arylesterase (AREase) activity. Since PON1 rates of phenyl acetate hydrolysis do not differ between *PON1*_{192Q} and R alloforms as they do for paraoxon hydrolysis (POase), AREase activity is considered a reliable measurement of PON1 enzyme levels. ELISA and Western blot based methods utilizing PON1 antibodies confirm a high correlation between measured PON1 levels and arylesterase activity ($r > 0.85$)[34,35].

In this study, PON1 enzyme activity towards 4 substrates (phenyl acetate, diazoxon (DZO), chlorpyrifos-oxon (CPO) and paraoxon (PO)) was measured in either serum or heparinized plasma samples using spectrophotometric methods as described previously [19]. Levels of arylesterase (AREase) activity were determined in a Molecular Devices SpectraMax[®] PLUS Microplate Spectrophotometer. Rate of formation of phenol was monitored every 15 sec (270 nm, ambient temperature) after the addition of 20 μ l (1:80 dilution) of plasma or serum to 200 μ l of a 3.26 mmol/l phenyl acetate solution (9 mmol/l Tris-HCL pH 8.0 0.9 mmol/l CaCl₂). For DZOase activity, hydrolysis of DZO was determined by measuring the rate of formation of 2-isopropyl-4-methyl-6-hydroxy pyrimidine (IMHP) when 20 μ l (1:10 dilution) of plasma were added to 200 μ l of 1 mmol/l DZO (2 M NaCl 0.1 mol/l Tris-HCL pH 8.5 2.0 mmol/l CaCl₂) at ambient temperature. Since AREase and DZOase assays were performed at ambient room temperature, their measurements may be subject to temperature fluctuations present in the laboratory environment. In contrast, CPOase and POase activities were measured at 37°C (controlled by SpectraMax) and therefore were less prone to temperature fluctuations in the laboratory. Readings were made every 15 sec at 310 nm and 405 nm, respectively. Buffers were prewarmed in a 37°C water bath. Formation of 3,5,6-trichloro-2-pyridinol (TCP) and p-nitrophenol was measured after addition of 20 μ l (1:20 and 1:10 dilution respectively) to 300 μ mol/l CPO or 1.2 mmol/l PO, respectively (2 mol/l NaCl 0.1 mol/l Tris-HCL pH 8.5 2.0 mmol/l CaCl₂). AREase, DZOase, and CPOase assays were carried out using UV transparent 96-well plates. Initial linear rates of hydrolysis (0–2 min) in mOD/min were converted to U/ml for AREase and U/l for DZOase, CPOase, and POase activities using the following molar extinction coefficients: 1.310 mmol/l•cm, 3.03 mmol/l•cm⁻¹, 5.56 mmol/l•, 18 mmol/l•cm, respectively. All samples were assayed in triplicate and the mean of the 3 values was used for subsequent analyses.

2.4 Statistical analysis

The CVs were calculated to summarize overall inter-laboratory variability. DZOase and POase activities were log transformed in all analyses to normalize their distributions. Pearson's correlation coefficient was calculated to examine correlations between PON1 activities measured at the UW laboratory vs the UCB laboratory; a paired *t*-test was used to determine differences between measurements made at the 2 laboratories. Additionally, Deming regression was used to explore associations between measurements made at the two labs and the Bland-Altman procedure was performed to determine their degree of agreement and produce difference plots. Similarly, to compare PON1 enzyme activity measured in serum and heparinized plasma, CV and Pearson's correlation coefficients were generated and Deming regression and the Bland Altman procedure were performed. A paired *t*-test was also used to evaluate differences between PON1 activities in serum versus plasma. Intra- and inter-assay

variability of lab control samples was described using CV. Linear regression models were used to determine the effect of changes in temperature (from 25°C) on PON1 enzymatic activity for AREase and DZOase, the 2 assays performed at ambient temperature. For this linear regression model, the dependent variable was percent change in enzyme activity (from that measured at 25°C) and the independent variable was the temperature difference from 25°C. Pearson's correlation analysis and linear mixed models were utilized for comparison of PON1 activity in CHAMACOS samples (n=95) assayed after 2 and 7 y of storage (-80°C). The linear mixed model allows for the evaluation of differences between measurements at years 2 and 7, while accounting for repeated measures on the same subjects and adjusting for assay temperature. CV was calculated to examine the effects of multiple freeze-thaw cycles (1-4) on laboratory volunteer samples. Linear mixed models were also used to compare the effects of storage duration on samples that had been thawed once between years 2 and 7 of storage and those that had not been thawed over that time. All statistical analyses were performed using STATA 9.0 (Statacorp, College Station, TX).

3. Results

3.1 Inter-laboratory variability

To confirm the overall reproducibility of the PON1 enzymatic assays between laboratories, a subset of 22 CHAMACOS samples were assayed in parallel both in the Holland laboratory at UCB, and at the Furlong laboratory at UW. We observed low variability between laboratories (Fig. 1). The average CV's for AREase and POase (for the 22 sample pairs) were 7% and 5%, respectively, and were comparable to intra-lab variability. Enzyme activity measures generated by the 2 laboratories were highly correlated. Pearson correlation coefficients for AREase, ln DZOase, CPOase, and ln POase were 0.97, 0.95, 0.96, 0.99 respectively ($p < 0.0005$ for all 4 PON1 assays). We did not detect a statistically significant difference between laboratories for AREase, CPOase, and ln POase activities (paired *t*-test). However, measurements of ln DZOase by the UCB laboratory were slightly lower (10%) than the UW laboratory (paired *t*-test; $p = 0.04$). The Deming regression analyses yielded similar results indicating slightly higher DZOase activity measurements at the UW laboratory. Comparison of the UW laboratory measurements (*y*) vs the UCB laboratory measurements (*x*) produced slopes (95% confidence interval in parentheses) of 0.95(0.84,1.06), 1.32(1.11,1.52), 1.05(0.89,1.21) and 0.97(0.91,1.02) and *y*-intercepts of 0.61(-6.95,8.17), -1.52(-2.53, -0.50), -0.28(-12.6,12.0), and 0.18(-0.17,0.53) for AREase, ln DZOase, CPOase, and ln POase, respectively. Bland-Altman plots for AREase and POase are shown in Figure 1.

3.2 Serum versus heparinized plasma in PON1 enzymatic assays

PON1 enzyme activity was evaluated in samples from 38 children from the IBD cohort for comparison of measurements in serum and heparinized plasma. The average intra-assay CV calculated from triplicate measurements of each sample was about 1% for both sample types in all 4 assays. The average CV between paired serum and plasma samples (for the same subject) was 15.3%, 15.3%, 14.7%, and 15.4% for AREase, DZOase, CPOase, and POase, respectively. In addition, PON1 enzyme activity in serum and plasma were highly correlated (Fig. 2). The Pearson's correlation coefficient was 0.72, 0.65, 0.75, and 0.94 for AREase, ln DZOase, CPOase, and ln POase, respectively ($p < 0.0005$ for all assays). We found that PON1 enzyme activities were consistently higher in serum than in heparinized plasma both by paired *t*-test analysis ($p < 0.01$) and Deming regression. The Deming regression equations for the difference between heparin plasma (*y*) and serum (*x*, 95% confidence interval in parentheses) were: $y = 1.02(0.67, 1.38)x - 17.3(-65.1, 30.6)$, $y = 1.12(0.66, 1.59)x - 1.31(-5.80, 3.19)$, $y = 1.10(0.77, 1.42)x - 1571.7(-4066.8, 923.4)$, and $y = 1.25(1.04, 1.46)x - 1.79(-3.21, -0.36)$ for AREase, lnDZOase, CPOase, and lnPOase, respectively. The average percent difference between PON1 activity measured in serum and plasma from the same individual was 21.6%,

22.2%, 23.5% and 26.1% for AREase, DZOase, CPOase, and POase, respectively. The absolute differences for AREase and In POase are shown in the Bland-Altman plots (Figs. 2B and D).

3.3 Intra- and inter-assay variability in plasma

Laboratory volunteer samples were collected in 2006 and multiple aliquots were prepared and stored at -80°C for use as internal control samples in >20 separate experiments over 2 y. In addition, within the same experiment, multiple aliquots of the same internal controls were prepared and each aliquot was assayed on a different plate. Within-assay variability as characterized by the average CV for within experiment measurements of lab control samples was relatively low. The CV was 4.3%, 2.8%, 2.1%, and 2.8% for AREase, DZOase, CPOase, and POase, respectively. As expected, the inter-assay variability was slightly higher than intra-assay variability. The average CV between assays was 7.5%, 5.7%, 7.6%, and 9.0% for AREase, DZOase, CPOase, and POase, respectively (Fig. 3).

3.4 Effects of temperature on AREase and DZOase activity

Lab volunteer samples were assayed multiple times at temperatures ranging from 24.7°C to 29.3°C . Enzyme activity in both assays increased with temperature (Fig. 4). Using linear regression analysis, we found that an increase of temperature by 1°C was associated with a 4.5% ($p<0.001$) and a 2.6% ($p<0.001$) increase in AREase and DZOase, respectively. Since temperature affected the assay results, we used the equations generated by linear regressions (Fig. 4) to predict AREase and DZOase values for a constant temperature of 25°C in subsequent analyses. As an alternative to using these temperature correction factors, we also found that including an assay temperature variable in the statistical model yielded similar results.

3.5 Effect of storage duration

To examine the effects of storage duration in samples stored for < 2 ys, we assayed multiple plasma aliquots from the same lab volunteer sample at different lengths of storage time (-80°C) ranging from 415 to 631 days. All 4 PON1 assays were performed on these aliquots a total of 17 times. We did not observe a significant association between sample storage duration and AREase, In DZOase, and CPOase activity. However, we did find a statistically significant decline in In POase activity with storage duration. The decrease was equivalent to 0.05% in POase activity per day of storage ($p=0.027$) and appeared linear for the particular range of time observed (days 415–631).

Plasma samples from 95 CHAMACOS mothers were assayed in 2002 at the UW laboratory (after 2 y of storage) [24,36] and then separate aliquots of the same samples that had not yet been thawed were assayed in 2007 after 2 y of storage in the UCB laboratory to determine the long term effects of storage duration. The measurements made after 2 y of storage were significantly correlated with measurements made after 7 y of storage for all 4 assays; however, the correlation was highest for POase activity. The Pearson's correlation coefficients were 0.51, 0.64, 0.55, and 0.79 for AREase, In DZOase, CPOase, and In POase respectively ($p<0.0005$ for all 4 assays). On average, enzyme activities were 17.1%, 39.4%, and 37.6% lower in the set of samples stored for 7 y as compared to those stored for 2 y ($P<0.001$ for all 3 assays) for AREase, CPOase, and POase respectively (Fig. 5). In contrast, DZOase activity was 22.9% higher ($p<0.0005$).

3.6 Effect of freeze-thaw cycles

We used multiple aliquots of plasma samples from 5 lab control volunteers to examine the effects of freeze-thaw cycles on PON1 enzyme activity in specimens stored at -80°C for < 2 y. Aliquots of each sample underwent between 1 and 4 freeze-thaw cycles before PON1 assays

were performed. We did not identify a significant change in PON1 activity for any of the 4 assays in samples experiencing a greater number of freeze-thaw cycles (Fig. 6). The inter-sample CV (freeze-thaw cycles) was 6.6%, 4.9%, 5.3%, and 6.0% for AREase, DZOase, CPOase, and POase, respectively. Thus, freeze-thaw cycles did not appear to affect PON1 enzyme activity assays in samples stored < 2 y.

In contrast, freeze-thaw cycles did affect PON1 enzyme activity in samples that had been stored for > 2 y. We assayed a subset of 27 CHAMACOS samples at years 2 and 7 (duration of storage at -80°C). Between years 2 and 7, these samples had undergone one additional freeze-thaw cycle. On average, PON1 enzyme activity was 25.7% lower ($p<0.0005$) for AREase and 14.2% higher for DZOase activity ($p=0.001$) after adjusting for temperature (Fig. 5). In comparison to the 95 CHAMACOS samples which had not been thawed between years 2 and 7, the thawed samples experienced a more dramatic decrease in AREase activity (17.1% for unthawed samples and 25.7% for thawed samples) that was statistically significant ($p<0.0005$). For In DZOase, both thawed and unthawed samples had increased activity at year 7. For CPOase and In POase activity, the additional freeze-thaw cycle did not significantly affect PON1 enzyme activity differences between samples stored for 2 and 7 y ($p=0.39$ and $p=0.87$, respectively) and the decrease in activity was comparable to unthawed samples (36.2% and 33.5%, respectively).

4. Discussion

In this study, we sought to characterize sources of technical variability for PON1 substrate-specific enzyme activity assays. We focused particularly on longitudinal studies, which can generate thousands of banked samples that are often analyzed after different periods of time have elapsed. We observed differences in measurable PON1 enzyme activity due to several sources: (1) use of heparinized plasma versus serum, (2) assay temperature, (3) duration of storage, and (4) freeze-thaw cycles; of these sources, duration of storage had the greatest impact on PON1 activity, particularly in samples stored for more than two years. We found relatively low intra- and inter-assay CVs (<10%) for control samples; intra- and inter-assay CV for POase were comparable to those previously reported in serum (3.5% and 2.7%, respectively) [22]. Additionally, we observed low inter-laboratory variability for samples run in parallel using identical reagents, software, and protocols at UCB and UW. These results establish good reproducibility of the PON1 enzymatic assays and support their suitability for use in epidemiologic studies. However, these sources of technical variability should be carefully considered in study design, subsequent statistical analysis, and interpretation of findings.

While a previous study of storage time on serum stored at -20°C reported no significant change in AREase activity over the course of 6 months [28], we observed lower activity in all assays except DZOase in samples stored for 7 y at -80°C . Similar results were reported in one study, which described a gradual decrease in serum POase activity in samples stored up to 4 y at -20°C [29] and in another which found that AREase, DZOase, and POase activity in plasma samples stored up to 6 y at -20°C decreased over time [37]. In longitudinal studies, it will be important to consider the effects of storage duration on PON1 activity, especially when comparing measurements from samples stored for varying lengths of time. Stenzel et al. [37] calculated a half-life of 9.5 y for POase activity stored at -20°C by fitting an exponential function to their data; thus it may be possible to adjust for storage duration in longitudinal studies by fitting a similar model.

Our analysis of long term storage duration compared PON1 enzyme activity measurements made in the UW laboratory to measurements made on the same samples 5 y later at the UCB laboratory. We must acknowledge that storage duration could not be isolated as the only source of variation since factors that may have differed between the 2 laboratories (i.e., possible

changes over time with respect to reagents, equipment, or procedures) may have also affected PON1 measurements. However, our inter-laboratory exercise established good comparability between the two laboratories for concurrent experiments (using the same protocols and reagents).

We did not observe any effect of multiple freeze-thaw cycles (up to 4) on PON1 enzyme activity in the UCB volunteer samples (storage duration up to 2 u). However, in samples stored up to 7 y (-80°C), freeze-thaw cycles resulted in decreased AREase and slightly increased DZOase activity. Similar to storage duration, multiple freeze-thaw cycles do not seem to impact PON1 enzyme activity as dramatically in one to two year old samples as compared to 7 y old samples. It is also important to note that the impact of freeze-thaw cycles was not consistent for all 4 substrate-specific assays, but mostly affected decreased AREase activity. One possible explanation is that unlike the 3 other substrate-specific PON1 assays, AREase assay uses an aryl ester substrate (phenyl acetate) rather than a phosphotriester (DZO, CPO, and PO). A site-directed mutagenesis study of PON1 demonstrated that the active sites for aryl esters and phosphotriesters are different [38]. Thus, it is not unexpected that the rates of hydrolysis of the different substrates could be differentially affected by storage conditions.

Temperature was positively correlated with AREase and DZOase activity. These two assays are often performed at ambient room temperature and thus may be affected when temperature fluctuations cannot be avoided in the laboratory environment. To minimize these effects, one could (1) adjust for temperature variation using statistical modeling or (2) ensure temperature stabilization by setting the microplate spectrophotometer temperature to some level slightly higher than typical room temperature.

Specimen type is another source of variability for PON1 enzyme activity. Some studies may only have certain specimens available for analyses; therefore it is important to determine what potential differences may arise when performing assays in varying specimen types such as serum or heparinized plasma. In our study, PON1 activity was higher in serum than plasma. However, enzyme activity in serum and heparinized plasma samples were highly correlated. Our results corroborated those reported by Brackley et al. [29], who also reported higher PON1 enzyme activity in serum versus plasma. They suggest that fibrin clots retain water, resulting in slightly higher serum concentrations. These differences between serum and plasma PON1 activity should be taken into account when designing a study and comparing values to those reported in other studies.

This study provided an opportunity to carefully examine challenges that may exist when using PON1 assays in longitudinal and pediatric disease studies. Utilizing samples from 2 pediatric cohorts, we were able to explore a comprehensive set of variability factors. Additionally, samples from these cohorts had been stored for long periods of time, allowing us to determine the effects of storage time over much greater durations than most previous studies.

PON1 has been implicated in many important pathways affecting health, including OP sensitivity and oxidative stress, and the number of studies of PON genotypes and activity is growing quickly [1,39–41]. Measurement of PON1 levels as determined by enzymatic assays is essential for determination of PON1 status and provides more information than PON1 genotype alone. Thus, use of PON1 enzymatic assays to characterize host variability may help clarify potential susceptibilities to major health endpoints. Our study demonstrates that results are relatively reproducible within and between assays and also between laboratories when using a standardized protocol. Sources of technical variability including temperature, storage duration, and freeze-thaw cycles should be monitored and minimized either via study design or through statistical adjustment. It is of particular concern for longitudinal studies, where long term specimen handling and storage are more likely to affect results.

Acknowledgements

We are grateful to the laboratory and clinical staff and participants of the CHAMACOS study and the Pediatric IBD consortium for their contributions. This publication was made possible by grant number R03 DK60617 from the National Institutes of Health (NIH), R826886 and R82670901 from the U.S. Environmental Protection Agency (EPA), R01ES012503-03, PO1 ES009605, ES09883, ES09601, and ES04696 from the National Institute of Environmental Health Science (NIEHS). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH and the EPA.

Abbreviations

PON1	Paraoxonase 1
HDL	high-density-lipoprotein
OP	organophosphate
IBD	inflammatory bowel disease
CHAMACOS	Center for Health Assessment of Mothers and Children of Salinas
UCB	University of California, Berkeley
UW	University of Washington, Seattle
DZO	diazoxon
CPO	chlorpyrifos-oxon
PO	paraoxon
AREase	arylesterase
IMHP	2-isopropyl-4-methyl-6-hydroxy pyrimidine
TCP	3,5,6-trichloro-2-pyridinol
CV	coefficient of variation
CI	confidence interval

References

1. Li HL, Liu DP, Liang CC. Paraoxonase gene polymorphisms, oxidative stress, and diseases. *J Mol Med* 2003;81:766–779. [PubMed: 14551701]
2. Costa LG, Cole TB, Vitalone A, Furlong CE. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005;352:37–47. [PubMed: 15653099]
3. DPR, Pesticide Use Report, Annual 2005. Sacramento, CA: Department of Pesticide Regulation, California Environmental Protection Agency; 2005.
4. Rosamond W, Flegal K, Friday G, et al. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007;115:e69–171. [PubMed: 17194875]
5. Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. *Free Radic Biol Med* 2004;37:1304–1316. [PubMed: 15454271]
6. Rosenblat M, Gaidukov L, Khersonsky O, et al. The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. *J Biol Chem* 2006;281:7657–7665. [PubMed: 16407304]
7. Tward A, Xia YR, Wang XP, et al. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation* 2002;106:484–490. [PubMed: 12135950]
8. Eskenazi B, Bradman A, Castorina R. Exposures of children to organophosphate pesticides and their potential adverse health effects. *Environ Health Perspect* 1999;107 (Suppl 3):409–419. [PubMed: 10346990]
9. Eskenazi B, Harley K, Bradman A, et al. Association of in utero organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. *Environ Health Perspect* 2004;112:1116–1124. [PubMed: 15238287]
10. Young JG, Eskenazi B, Gladstone EA, et al. Association between in utero organophosphate pesticide exposure and abnormal reflexes in neonates. *Neurotoxicology* 2005;26:199–209. [PubMed: 15713341]
11. Berkowitz GS, Wetmur JG, Birman-Deych E, et al. In utero pesticide exposure, maternal paraoxonase activity, and head circumference. *Environ Health Perspect* 2004;112:388–391. [PubMed: 14998758]
12. Chen J, Chan W, Wallenstein S, Berkowitz G, Wetmur JG. Haplotype-phenotype relationships of paraoxonase-1. *Cancer Epidemiol Biomarkers Prev* 2005;14:731–734. [PubMed: 15767359]
13. Lawlor DA, Gaunt TR, Hinks LJ, et al. The association of the PON1 Q192R polymorphism with complications and outcomes of pregnancy: findings from the British Women's Heart and Health cohort study. *Paediatr Perinat Epidemiol* 2006;20:244–250. [PubMed: 16629699]
14. Erlich PM, Lunetta KL, Cupples LA, et al. Polymorphisms in the PON gene cluster are associated with Alzheimer disease. *Hum Mol Genet* 2006;15:77–85. [PubMed: 16319130]
15. Zintzaras E, Hadjigeorgiou GM. Association of paraoxonase 1 gene polymorphisms with risk of Parkinson's disease: a meta-analysis. *J Hum Genet* 2004;49:474–481. [PubMed: 15368102]
16. Bhattacharyya T, Nicholls SJ, Topol EJ, et al. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *Jama* 2008;299:1265–1276. [PubMed: 18349088]
17. Browne RW, Koury ST, Marion S, Wilding G, Muti P, Trevisan M. Accuracy and biological variation of human serum paraoxonase 1 activity and polymorphism (Q192R) by kinetic enzyme assay. *Clin Chem* 2007;53:310–317. [PubMed: 17185369]
18. Jarvik GP, Rozek LS, Brophy VH, et al. Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1(192) or PON1(55) genotype. *Arterioscler Thromb Vasc Biol* 2000;20:2441–2447. [PubMed: 11073850]
19. Richter RJ, Furlong CE. Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics* 1999;9:745–753. [PubMed: 10634137]
20. Krisch K. Enzymatic hydrolysis of diethyl-p-nitrophenylphosphate (E600) by human serum. *Z Clin Chem* 1968;6:41–45.

21. Marton A, Kalow W. Studies on aromatic esterase and cholinesterase of human serum. *Can J Biochem Physiol* 1959;37:1367–1373. [PubMed: 14421868]
22. Charlton-Menys V, Liu Y, Durrington PN. Semiautomated method for determination of serum paraoxonase activity using paraoxon as substrate. *Clin Chem* 2006;52:453–457. [PubMed: 16423902]
23. Richter, R.; Jampsa, R.; Jarvik, G.; Costa, LG.; Furlong, CE. Determination of Paraoxonase 1 (PON1) Status and Genotypes at Specific Polymorphic Sites. In: Maines, MD.; Costa, LG.; Reed, DJ.; Hodgson, E., editors. *Current Protocols in Toxicology*. NY, NY: John Wiley and Sons; 2004. p. 4.12.11-14.12.19.
24. Furlong CE, Holland N, Richter RJ, Bradman A, Ho A, Eskenazi B. PON1 status of farmworker mothers and children as a predictor of organophosphate sensitivity. *Pharmacogenet Genomics* 2006;16:183–190. [PubMed: 16495777]
25. Cole TB, Jampsa RL, Walter BJ, et al. Expression of human paraoxonase (PON1) during development. *Pharmacogenetics* 2003;13:357–364. [PubMed: 12777966]
26. Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol Biomarkers Prev* 2007;16:1954–1965. [PubMed: 17932342]
27. Rundle AG, Vineis P, Ahsan H. Design options for molecular epidemiology research within cohort studies. *Cancer Epidemiol Biomarkers Prev* 2005;14:1899–1907. [PubMed: 16103435]
28. Nakanishi M, Takunami Y, Maruyama T, et al. The ratio of serum paraoxonase/arylesterase activity using an improved assay for arylesterase activity to discriminate PON1(R192) from PON1(Q192). *J Atheroscler Thromb* 2003;10:337–342. [PubMed: 15037822]
29. Brackley M, Carro-Ciampi G, Stewart DJ, Lowden JA, Ray AK, Kalow W. Stability of the paraoxonase phenotyping ratio in collections of human sera with differing storage times. *Res Commun Chem Pathol Pharmacol* 1983;41:65–78. [PubMed: 6312517]
30. Holland N, Harmatz P, Golden D, et al. Cytogenetic damage in blood lymphocytes and exfoliated epithelial cells of children with inflammatory bowel disease. *Pediatr Res* 2007;61:209–214. [PubMed: 17237724]
31. Heyman MB, Kirschner BS, Gold BD, et al. Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry. *J Pediatr* 2005;146:35–40. [PubMed: 15644819]
32. Eskenazi BBA, Gladstone EA, Jaramillo S, Birch K, Holland NT. CHAMACOS, a longitudinal birth cohort study: lessons from the fields. *J Childrens Health* 2003;1:3–27.
33. ISBER. 2008 Best Practices for Repositories: Collection, Storage, Retrieval and Distribution of Biological Materials for Research. *Cell Preservation Technology* 2008;6:3–58.
34. Connolly PW, Maguire GF, Picardo CM, Teiber JF, Draganov D. Development of an immunoblot assay with infrared fluorescence to quantify paraoxonase I in serum and plasma. *J Lipid Res* 2008;49:245–250. [PubMed: 17906223]
35. Kujiraoka T, Oka T, Ishihara M, et al. A sandwich enzyme-linked immunosorbent assay for human serum paraoxonase concentration. *J Lipid Res* 2000;41:1358–1363. [PubMed: 10946025]
36. Holland N, Furlong C, Bastaki M, et al. Paraoxonase polymorphisms, haplotypes, and enzyme activity in Latino mothers and newborns. *Environ Health Perspect* 2006;114:985–991. [PubMed: 16835048]
37. Stenzel J, Worek F, Eyer P. Preparation and characterization of dialkylphosphoryl-obidoxime conjugates, potent anticholinesterase derivatives that are quickly hydrolyzed by human paraoxonase (PON1192Q). *Biochem Pharmacol* 2007;74:1390–1400. [PubMed: 17714697]
38. Khersonsky O, Tawfik DS. The histidine 115-histidine 134 dyad mediates the lactonase activity of mammalian serum paraoxonases. *J Biol Chem* 2006;281:7649–7656. [PubMed: 16407305]
39. van Himbergen TM, van Tits LJ, Roest M, Stalenhoef AF. The story of PON1: how an organophosphate-hydrolysing enzyme is becoming a player in cardiovascular medicine. *Neth J Med* 2006;64:34–38. [PubMed: 16517986]
40. James RW. A long and winding road: defining the biological role and clinical importance of paraoxonases. *Clin Chem Lab Med* 2006;44:1052–1059. [PubMed: 16958594]
41. Marchegiani F, Marra M, Olivieri F, et al. Paraoxonase 1: genetics and activities during aging. *Rejuvenation Res* 2008;11:113–127. [PubMed: 18279029]

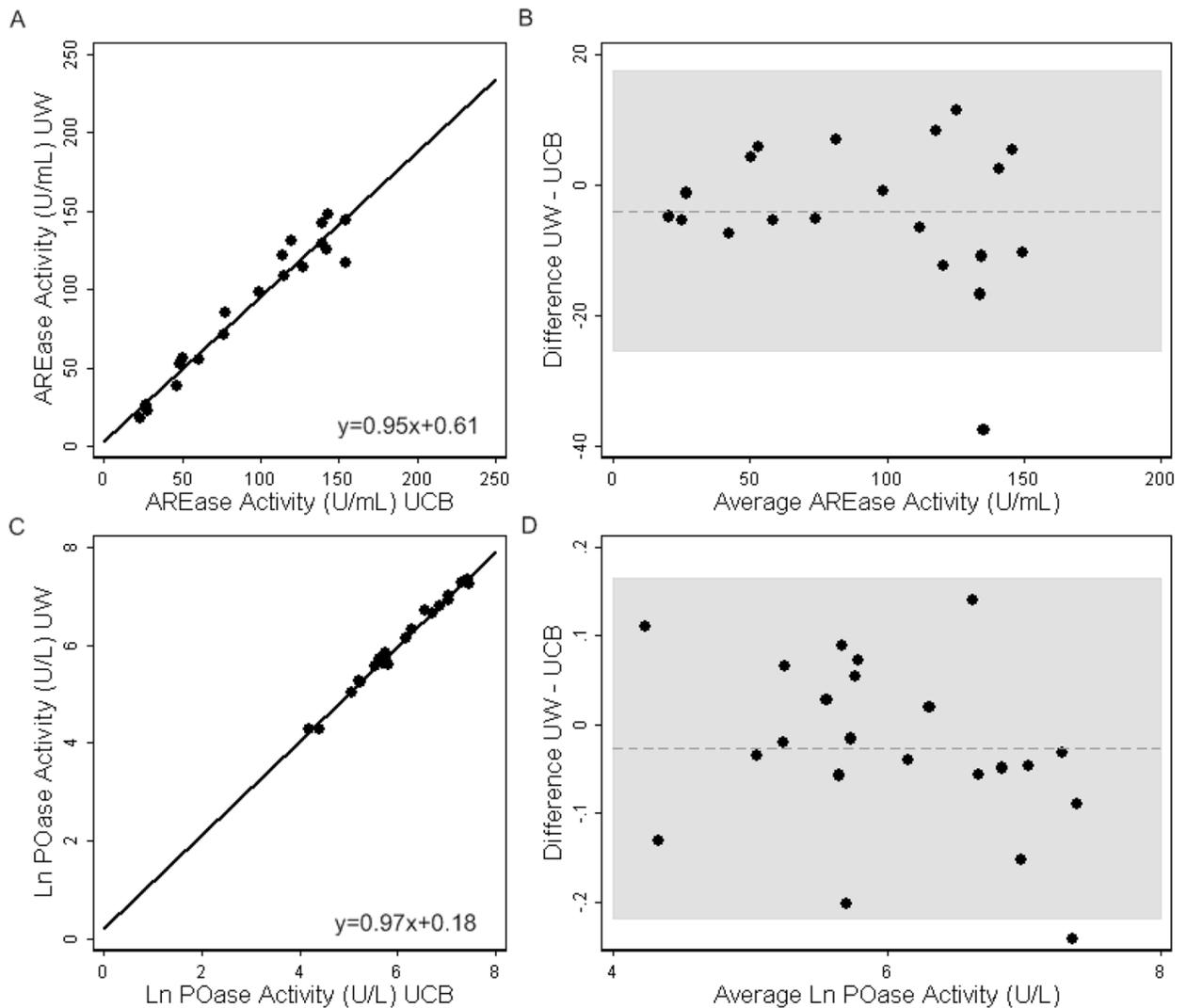


Figure 1.

Inter-laboratory variability of AREase (A) and POase (C) assays. PON1 assays were performed in parallel on the same 22 samples at the University of California, Berkeley (UCB), and the University of Washington, Seattle (UW), and using the same reagents, software, and protocols. Deming regression was used to determine the association between measurements made at each laboratory. Bland-Altman plots are shown for AREase (B) and Ln POase (D). The dotted line represents the mean difference between laboratory measurements (-4.0 and -0.03 for AREase and Ln POase, respectively) and the shaded region represents the 95% confidence intervals for this difference. For AREase, CV was 7% and the Pearson's correlation coefficient (R) was 0.97. For POase, CV was 5%. R for Ln POase was 0.99.

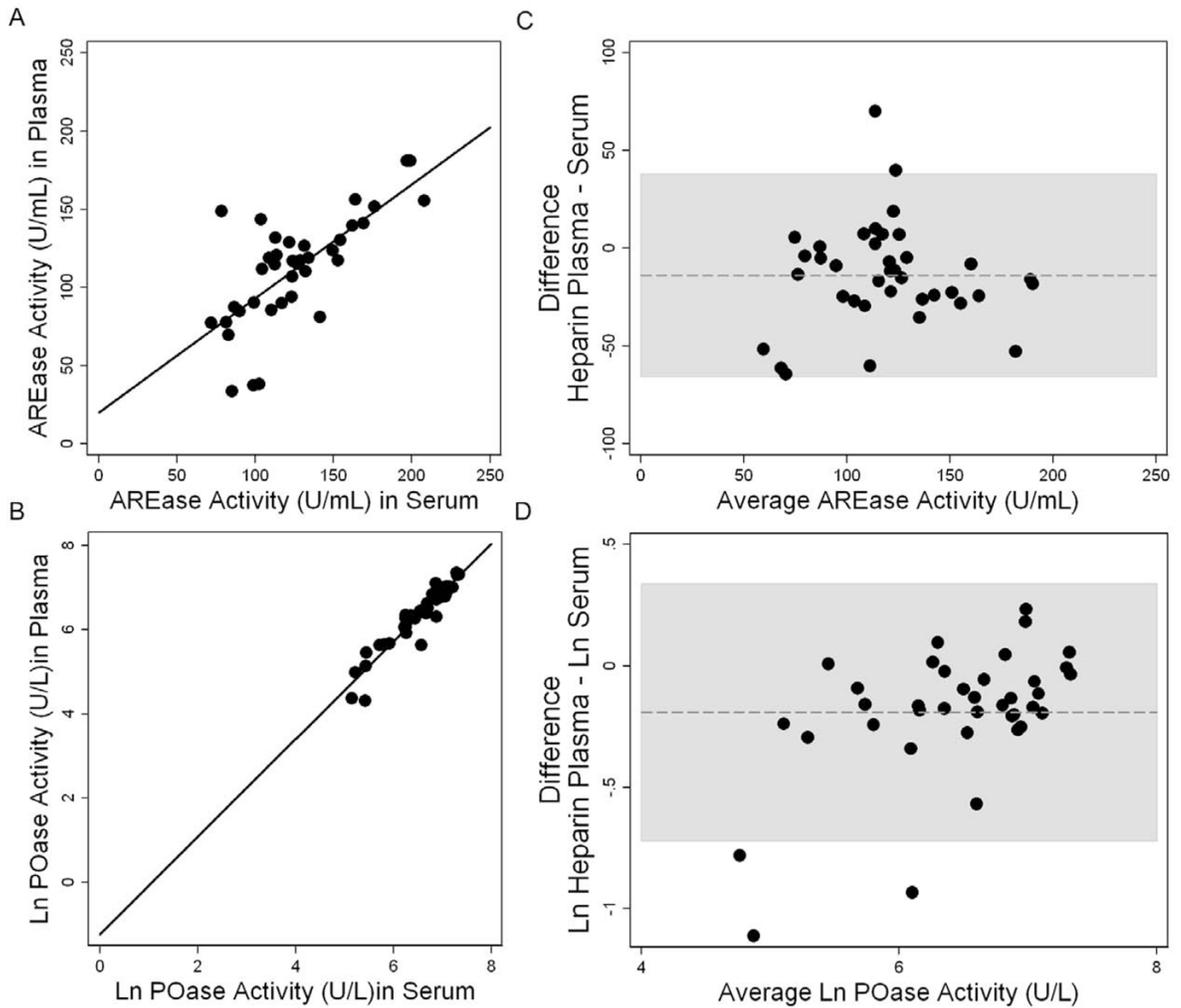


Figure 2.

PON1 enzyme activity assays in serum versus heparinized plasma. AREase (A,B) and Ln POase (C,D) activity were measured in serum and sodium heparin plasma in children (n=38). Deming regression was used to determine the association between measurements in serum and in plasma. Bland-Altman plots are shown for AREase (B) and Ln POase (D). The dotted line represents the mean difference between serum and plasma measurements (-13.8 and -0.19 for AREase and Ln POase, respectively) and the shaded region represents the 95% confidence interval for this difference. The Pearson's correlation coefficient was 0.72 ($p < 0.0005$) and 0.94 ($p < 0.0005$) for AREase and Ln POase, respectively.

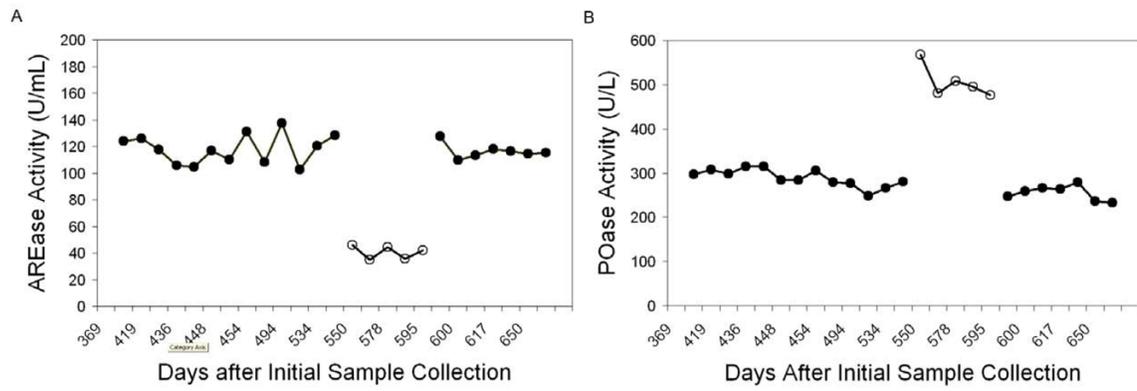


Figure 3. Inter-assay variability of PON1 enzyme activity in laboratory control plasma samples. Plasma samples were collected from laboratory volunteers in 2006 and multiple aliquots were made before storing at -80°C . Laboratory control sample 1 (●) and laboratory control 2 (○) were repeatedly assayed to measure inter-assay variability for all 4 PON1 substrates. The CV between assays was 7.5% for AREase (A) and 9.0% for POase (B).

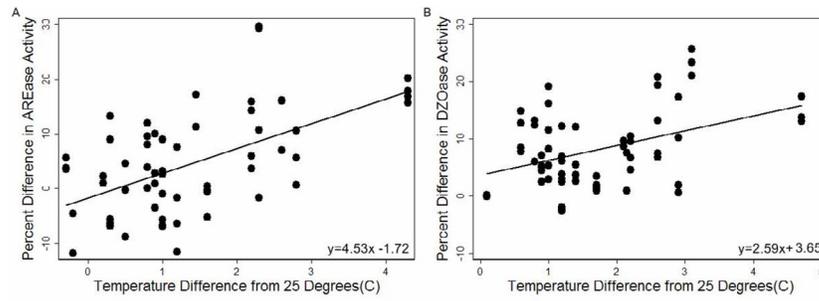


Figure 4. Effects of ambient temperature on PON1 enzyme activity assays. Both AREase (A) and DZOase (B) activities increased with temperature ($p < 0.001$ for both). Linear regression models were used to determine the relative percent change in AREase and DZOase activity per degree deviation from 25°C. Enzyme activity increased 4.5% and 2.6% per degree increase for AREase and DZOase, respectively.

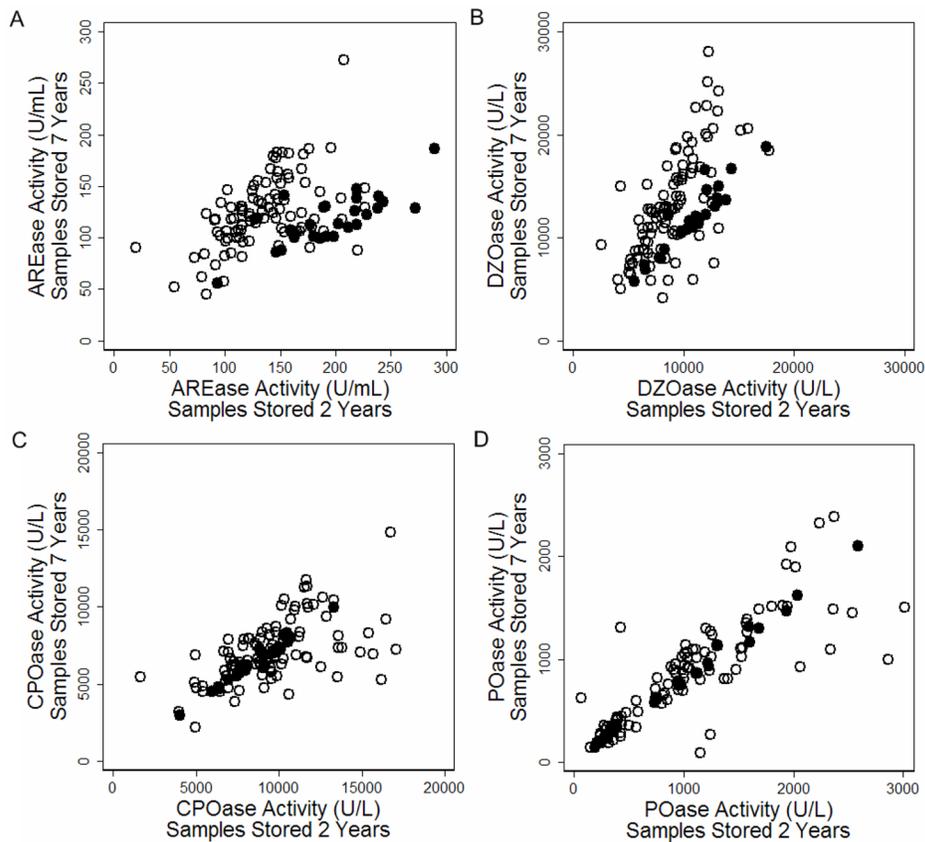


Figure 5.

For 95 subjects (\bullet), all 4 PON1 enzyme activity assays, AREase (A), In DZOase (B), CPOase (C), and In POase (D) were run after storage at -80°C at 2 and 7 y. Measures of all 4 enzyme activities were significantly correlated between samples from the same subjects run after 2 and 7 y of storage at -80°C . Correlation coefficients were 0.51, 0.64, 0.55, and 0.79, respectively ($p < 0.0005$ for all 4 enzyme activity assays). For a second subset ($n=27$, \circ), samples were also run after storage at 2 and 7 y. However, these samples had been through an additional freeze-thaw cycle before being rerun at 7 y of storage. Correlation coefficients for these previously thawed samples were 0.75, 0.95, 0.98, and 0.99 respectively ($p < 0.0005$ for all 4 enzyme activity assays).

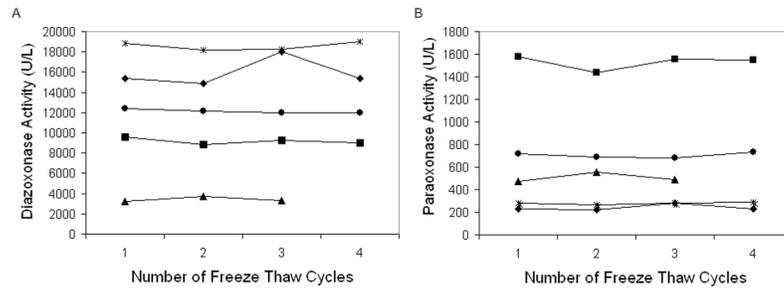


Figure 6. Effect of freeze-thaw cycles on PON1 enzyme activity. Five lab control samples were frozen at -80°C (sample 1 ◐; sample 2 ♦; sample 3 ◑; sample 4 ■; sample 5 ▲) and underwent 1–4 freeze-thaw cycles over 2 weeks. Average within-sample CV (%) was 6.5, 4.9, 5.3, and 6.0 for AREase, DZOase, CPOase, and POase, respectively.