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Short Communication

Covariation of Human Microsomal Protein Per Gram of Liver with Age: Absence of Influence of Operator and Sample Storage May Justify Interlaboratory Data Pooling

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ABSTRACT:

Scaling of metabolic clearance values from liver microsomal data or recombinantly expressed cytochrome P450 enzymes to predict human hepatic clearance requires knowledge of the amount of microsomal protein per gram of liver (MPPGL). Identification of physiological covariates of MPPGL requires analysis of values from large diverse populations, which necessitates pooling of data from numerous sources. To ensure compatibility between results obtained within and between studies, the impact of interoperator differences and sample storage on values of MPPGL was investigated. With use of triplicate samples from one liver (HL86), no statistically significant difference was detected between values of MPPGL prepared from samples stored at −80°C (23.5 ± 1.2 mg g^{-1}) and those determined using fresh tissue (21.9 ± 0.3 mg g^{-1}). Although there was a significant difference in the yield of microsomal protein obtained from another liver sample (HL43) by three different operators (17 ± 1, 19 ± 2, and 24 ± 1 mg g^{-1}; $p = 0.004$, analysis of variance), no difference was observed in the estimated MPPGL after application of appropriate correction factors for each operator (28 ± 1, 30 ± 5, and 31 ± 4 mg g^{-1}). The result provided justification for pooling reported values of MPPGL for use in covariate analysis. Investigation of the relationship between age and MPPGL provided preliminary evidence that MPPGL values increase from birth to a maximum of 40 mg g^{-1} [95% confidence interval for the geometric mean (95% CI $mean_{geo}$): 37–43 mg g^{-1}] at approximately 28 years followed by a gradual decrease in older age (mean of 29 mg g^{-1} at 65 years; 95% CI $mean_{geo}$: 27–32 mg g^{-1}). Accordingly, appropriate age-adjusted scaling factors should be used in extrapolating in vitro clearance values to clinical studies.

The use of in vitro-in vivo extrapolation (IVIVE) of metabolic data in the prediction of population clearance has become an important tool in both discovery and preclinical phases of the drug development process (Rostami-Hodjegan and Tucker, 2007). Scaling of metabolic rate constants derived using microsomal protein (MSP) isolated from human livers or those from recombinantly expressed cytochrome P450 enzymes requires knowledge of the amount of microsomal protein per gram of liver (MPPGL) among other scaling factors (Barter et al., 2007). The most commonly used value of MPPGL in human IVIVE is 45 mg g^{-1} (Obach, 1997; Soars et al., 2002; Andersson et al., 2004; Uchaipichat et al., 2006) reported in a review by Houston (1994). However, this value is not obtained from human livers; instead it is a combination of data generated using rat tissue from a number of sources (Joly et al., 1975; Lin et al., 1978; Baarnhielm et al., 1986; Chiba et al., 1990). Several values of MPPGL determined using human tissue have been reported in the literature, and a detailed review of these studies has been the focus of a recent consensus article on the most appropriate values of MPPGL for use in IVIVE alongside other scaling factors such as human hepatocellularity (Barter et al., 2007). Collation of values of MPPGL from five studies (114 observations; age range 11–80 years, median 48 years; 47 females) has indicated a weak but statistically significant inverse relationship between MPPGL and donor age (Barter et al., 2007).

Barter et al. (2007) assumed that different experimental procedures and technical staff carrying out the exercise ("operators") would not introduce bias into their analysis. However, these effects have not been assessed systematically. Assessing the operator effect also has implications for within-laboratory pooling of data. The preparation and analysis of replicate samples, which are required to differentiate between methodological and true biological variability in values of MPPGL, is time-consuming (2 days/liver). To increase output, processing of samples may be performed by more than one individual (operator); however, this will require an indication of consistency between estimates of MPPGL by these operators.

In addition, determination of values of MPPGL from fresh tissue from large numbers of donors is hampered by the infrequent supply of human tissue and logistical problems associated with immediate analysis of the samples when they arrive at the laboratory. In theory, the use of tissue stored at −80°C solves this problem. However, the use of frozen samples assumes maintenance of protein structure and function through the freeze-thaw process. Thus, to ensure adequate compatibility between the results obtained within and between differ-

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ABBREVIATIONS: IVIVE, in vitro-in vivo extrapolation; MSP, microsomal protein; MPPGL, microsomal protein per gram of liver; P450, cytochrome P450; ANOVA, analysis of variance; AIC, Akaike's information criterion; 95% CI $mean_{geo}$, 95% confidence interval for the geometric mean; 95% CI obs, 95% confidence interval for the likelihood of observations.

ent studies (with the ultimate goal of combining data in large-scale analysis) two major questions relating to experimental variables must be addressed: 1) Does the operator involved in the preparation and analyses of samples have an impact on values of MPPGL derived from the experiments? and 2) Does the use of "frozen tissue" in the determination of values of MPPGL produce results comparable with those obtained from fresh tissue?

In the current report, we provide evidence for consistency of MPPGL values obtained from different operators and fresh versus frozen liver samples. In addition, we reanalyze the influence of age on MPPGL by incorporating neonatal and pediatric MPPGL values into the pool of the previously reported data set (Barter et al., 2007).

Materials and Methods

Reagents. All laboratory chemicals were purchased from either Sigma-Aldrich (Dorset, UK) or Bio-Rad (Herts, UK).

Source of Human Tissue. Samples of adult human liver were obtained from the collection of human liver samples held within the Academic Unit of Clinical Pharmacology at the University of Sheffield as described previously (Wilson et al., 2003). Samples of pediatric human liver were obtained from Vitron (Tucson, AZ) by the Environmental Protection Agency and shipped to the University of Sheffield for analysis of microsomal protein content.

Study 1: Fresh Versus Frozen Tissue. Values of MPPGL were determined in the "same liver" sample (HL84), immediately after surgical excision ("fresh") and again from the same liver tissue after storage for 1 week at −80°C ("frozen"). All samples were from the same lobe. After extraction, liver samples were handled as is routine in our laboratory: placement on ice within 10 min of excision, dissection into approximately 3-g amounts followed either by immediate homogenization (within an additional hour; fresh) or flash freezing in liquid nitrogen and storage at −80°C (frozen). Homogenate and microsomes were prepared, and P450 and total protein contents were measured as described previously (Wilson et al., 2003). The P450 content of samples was determined by dithionite difference spectroscopy according to the method of Matsubara et al. (1976), and MSP was determined using the method of Bradford (1976). After 1 week, 3 g of tissue was thawed, and homogenate and microsomal samples were prepared and assayed for P450 and total protein contents. Values of MPPGL corrected for the fraction of microsomal protein lost during centrifugation were calculated using paired homogenate and microsomal samples with the following equations:

$$\text{Fractional loss of MSP} = 1 - \left(\frac{\text{P450}_{\text{microsomal}}\ (\text{nmols})}{\text{P450}_{\text{homogenate}}\ (\text{nmols})}\right) \quad (1)$$

$$\text{MPPGL (mg g}^{-1}) = \frac{\text{Yield of microsomal protein (mg g}^{-1})}{(1 - \text{fractional loss of MSP})} \quad (2)$$

Levels of microsomal yield and values of MPPGL in the two samples were then compared using an unpaired t test (Data Analysis Toolpak, Microsoft Office Excel 2003; Microsoft, Richmond, WA). The significance of relationships was assessed by evaluation of p values.

Study 2: Interoperator Variability. To investigate the effect of multiple operators on the determination of values of MPPGL, homogenate and microsomal samples were prepared in triplicate from tissue from a single donor (HL43) by three different individuals (operators) following the same protocol and using the same instrumentation (as described previously by Wilson et al., 2003) on the same day. Repeated measurements of the liver sample by each operator allowed the estimation of interoperator variability in MPPGL. Differences in MPPGL between operators were assessed using one-way ANOVA (SPSS version 12; SPSS Inc., Chicago, IL).

Study 3: Assessing the Effect of Age on MPPGL from Birth to Old Age. Homogenate and microsomes were prepared from liver tissue from four Caucasian pediatric donors (three female; 2, 4, 9, and 13 years). Evaluation of the literature identified one study reporting values of MPPGL (Pelkonen et al., 1973) from 11 fetal samples (age since conception/developmental age not provided). Values of MPPGL from the 4 pediatric samples determined in this study and the 11 fetal samples from Pelkonen et al. (1973) were incorporated into the meta-analysis carried out previously by Barter et al. (2007) (n = 114 livers; 11–80 years of age; 43 females).

Because the Kolmogorov-Smirnov test showed that the data were compatible with a log-normal distribution the assessment of model fit was performed on log-transformed values of MPPGL. After the incorporation of pediatric and fetal liver samples to the data set reported previously (Barter et al., 2007), five models, including the model described by Barter et al. (2007), were assessed (Fig. 1) to best explain the relationship between donor age and MPPGL (GraphPad Prism 5; GraphPad Software Inc., San Diego, CA).

Model 1 was the model used in Barter et al. (2007). Models 2, 3, and 4 were extensions of model 1 (i.e., nested in model 1). However, model 5 had a different structure. Therefore, the F test was not appropriate for comparison of the models and hence AIC was used (Gabrielsson and Weiner, 2007).

Results

Study 1: Fresh Versus Frozen Tissue. No significant difference ($p > 0.05$) was detected in either homogenate (mean ± S.D.: fresh, 11.5 ± 0.7 nmol g^{-1} of liver; frozen, 11.2 ± 0.6 nmol g^{-1} of liver) or microsomal (fresh, 0.52 ± 0.03 nmol/mg microsomal protein; frozen, 0.48 ± 0.02 nmol/mg microsomal protein) P450 content and hence values of MPPGL prepared from liver samples stored at −80°C (23.5 ± 1.2 mg g^{-1}) and those determined using fresh tissue (21.9 ± 0.3 mg g^{-1}).

Study 2: Interoperator Variability. Despite using the same liver, same protocol, and same instrumentation, significant differences in microsomal protein yield were found among operators (mean ± S.D.: 17 ± 1, 19 ± 2, and 24 ± 1 mg g^{-1}; $p = 0.004$, ANOVA) (Fig. 2A). Significant differences in the percentage of microsomal protein lost during the preparation process were also observed (38 ± 3, 37 ± 6, and 22 ± 5%; $p = 0.01$, ANOVA) (Fig. 2B). However, no significant difference in estimated values of MPPGL corrected for losses in microsomal protein during preparation was observed, indicating that higher yields of microsomal protein were associated with lower losses of microsomal protein during preparation (28 ± 1, 30 ± 5, and 31 ± 4 mg g^{-1}; $p = 0.64$, ANOVA) (Fig. 2C). Post hoc analysis of the data (Tukey's b) indicated microsomal losses determined by operators 1 and 2 (38 and 37%) to be significantly different from that determined by operator 3 (22%; $p < 0.05$). Thus, accounting for interoperator differences in yield by application of a recovery factor (1 − fraction of microsomal protein lost) resulted in comparable values of MPPGL.

Study 3: Assessing the Effect of Age on MPPGL from Birth to Old Age. The geometric mean value of MPPGL corrected for loss of protein incurred during preparation for the four pediatric livers was 28 mg g^{-1} (range 23–30 mg g^{-1}). A fetal MPPGL value of 26 mg g^{-1} was calculated from data reported by Pelkonen et al. (1973).

The log-linear model reported by Barter et al. (2007) is shown in Fig. 1A, and MPPGL values from the pediatric and fetal samples are overlaid. Visual inspection of the overlaid data indicates a systematic departure and hence inadequacy of the previous model to describe MPPGL values at ages lower than those examined in the earlier meta-analysis. Graphic representation of the fit of the five models to the extended data set (n = 129 livers) is provided in Fig. 1, B to F. Incorporation of the additional samples from younger donors into the data set indicated a nonmonotonic relationship between age and MPPGL with values increasing from birth to a maximum of 40 mg g^{-1} around 28 years (95% CI mean$_{geo}$: 37–43 mg g^{-1}; 95% CI obs 19–85 mg g^{-1}) followed by a gradual decrease in older age (mean of 29 mg g^{-1} at 65 years; 95% CI mean$_{geo}$: 27–32 mg g^{-1}; 95% CI obs: 14–63 mg g^{-1}). Having disregarded model 1 as an appropriate model, a combination of comparison of AIC and visual inspection for the remaining models indicated that model 4 provided the best (most parsimonious) fit. This was mainly due to the ability of the model to generate physiologically relevant values of MPPGL even at the ex-

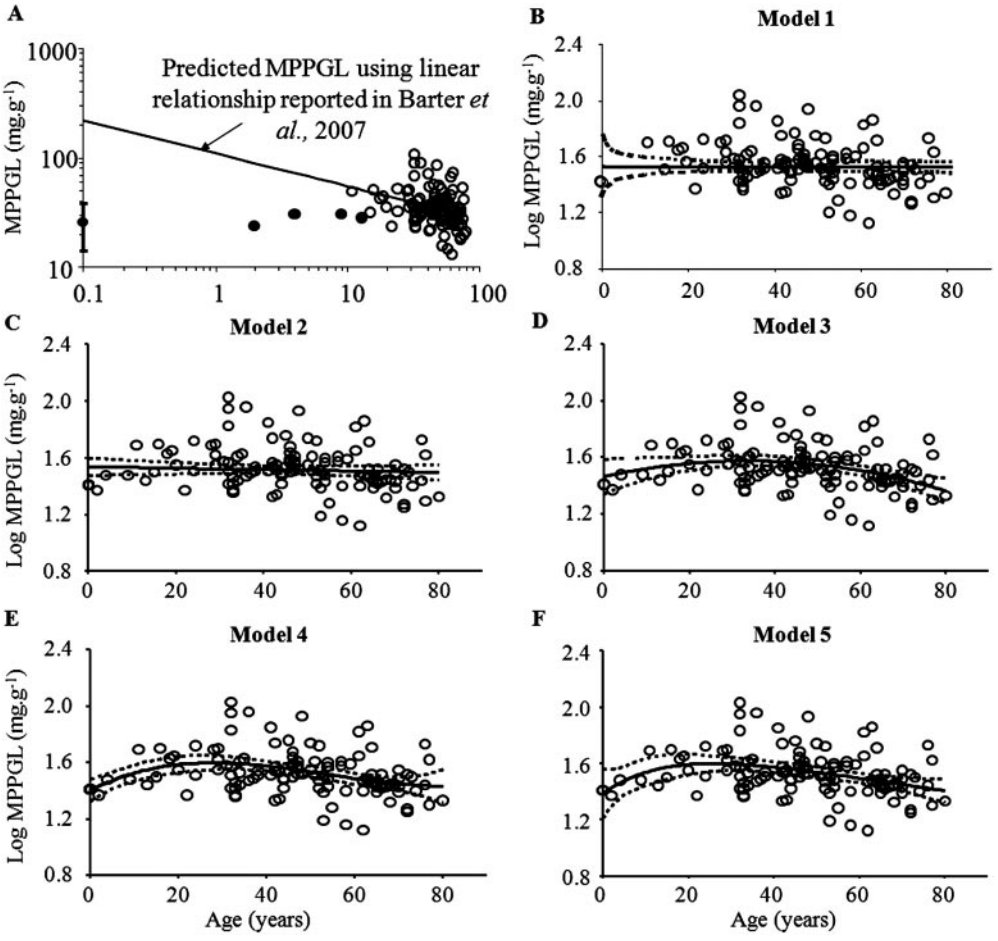

FIG. 1. A, relationship between age and MPPGL adapted from Barter et al. (2007). ○, original data set of 114 livers reported by Barter et al. (2007); ●, additional livers incorporated in the updated data set of 129 livers. Error bars represent the S.D. of the 11 fetal samples investigated by Pelkonen et al. (1973). B to F, relationship between age and MPPGL described using models 1 through 5. Extended data set of 129 livers. Dotted lines indicate the 95% confidence interval of the mean. B, model 1: $MPPGL = 10^{C0+C1\times \log age}$; C0 = 2.038, Cl = −0.3048. C, model 2: $MPPGL = 10^{C0+C1\times age}$; C0 = 1.540, C1 = −0.00049. D, model 3: $MPPGL = 10^{C0+C1\times age+C2\times age^2}$; C0 = 1.434, C1 = 0.0080, C2 = −0.00011. E, model 4: $MPPGL = 10^{C0+C1\times age+C2\times age^2+C3\times age^3}$; C0 = 1.407, C1 = 0.0158, C2 = −0.00038, C3 = 0.000024. F, model 5: $MPPGL = 10^{C0\times(\exp -C1\times age)+C2\times(\exp -C3\times age)}$; C0 = 1.835, C1 = 0.0033, C2 = −0.4465, C3 = 0.0639.

tremes of the population. The probability of model 4 being the correct model over models 2, 3, and 5 was 100, 25, and 5% more likely, respectively (differences in AIC: model 2 versus 4, 17.0; model 3 versus 4, 1.03; and model 4 versus 5, 0.21). Coefficient values for all of the models are provided in Fig. 1. The correlation between predicted versus observed values of MPPGL determined using model 4 was statistically significant ($p = 0.0004$). The following equation was used to calculate age-related values of MPPGL from birth to adult (Fig. 3):

$$MPPGL\ (mg\ g^{-1}) = 10^{(1.407+0.0158\times age-0.00038\times age^2+0.0000024\times Age^3)}$$

Discussion

A key point for consideration when one is determining values of hepatic scaling factors such as MPPGL is the quality of liver tissue used in their estimation. The P450 content of tissue left at 25°C for 6 h has been shown to be significantly reduced compared with the content of tissue maintained in a chilled environment (Yamazaki et al., 1997). Lengthy postperfusion cold time of liver tissue may also reduce the P450 activity of prepared microsomes (Lipscomb and Garrett, 1998). Therefore, the length of time from tissue extraction to placement in chilled medium should be kept to a minimum. In the current study, the delay between extraction and transfer to ice was minimized to 10 min. The infrequent supply of fresh human liver tissue has led to the establishment of collections of frozen tissue samples in many institutions, including the institution at which the present study was performed. Here, samples of human tissue or tissue fractions may be stored at −80°C for several years, providing a constant supply of human tissue. However, when one is using frozen tissue in the determination of MPPGL, the effect of storage on levels of P450 and total protein should be considered. Although the effect of sample storage on values of MPPGL has yet to be investigated, several studies have assessed the effect of storage on human liver microsomal P450 content (von Bahr et al., 1980; Powis et al., 1988; Pearce et al., 1996). Overall, very little difference in the microsomal protein content of fresh and frozen samples (10 mg g^{-1} fresh versus 12 mg g^{-1} frozen) (von Bahr et al., 1980) was observed. Powis et al. (1988) found no change in the microsomal P450 content (nanomoles per milligram) of liver samples stored either as microsomes, homogenate, or liver pieces at −80°C for up to 1 year. However, studies carried out by Pearce et al. (1996) showed that the P450 content of microsomes prepared from frozen liver was approximately 30% less than that in microsomes prepared from fresh liver. No increases in levels of the degraded, inactive form of P450, CYP420, were observed compared with those from fresh tissue, indicating that P450 degradation was not the cause of the reduction in P450 content. It is more likely that the increase in total MSP observed in microsomes prepared from frozen tissue was due to contamination by additional proteins such as hemoglobin, potentially resulting in the generation of artificially high values of MPPGL. Details on handling of the tissue after extraction, such as washing of samples to remove excess blood to minimize heme contamination, were not provided by Pearce et al. (1996). In the current study we only used samples ($n = 3$) from a single donor; however, we found no significant difference in either the homogenate (nanomoles per gram of liver)

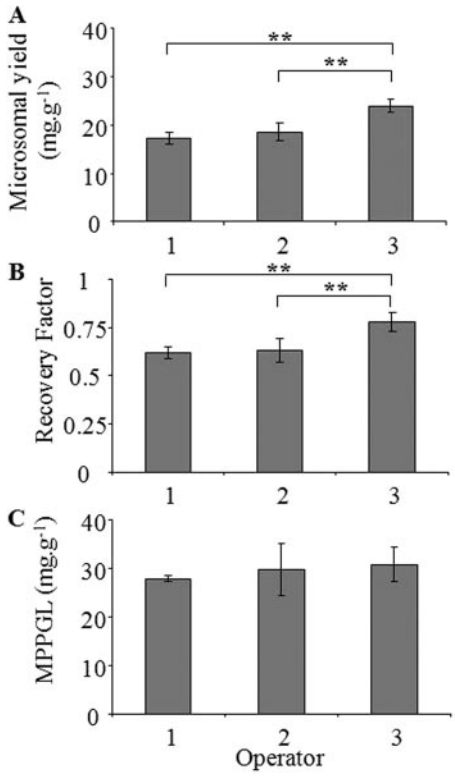

FIG. 2. The effect of multiple operators on microsomal protein yield (A), microsomal loss during preparation (B), and values of MPPGL corrected for loss (C). n = 3 determinations per operator ± S.D. **, $p < 0.05$; ANOVA.

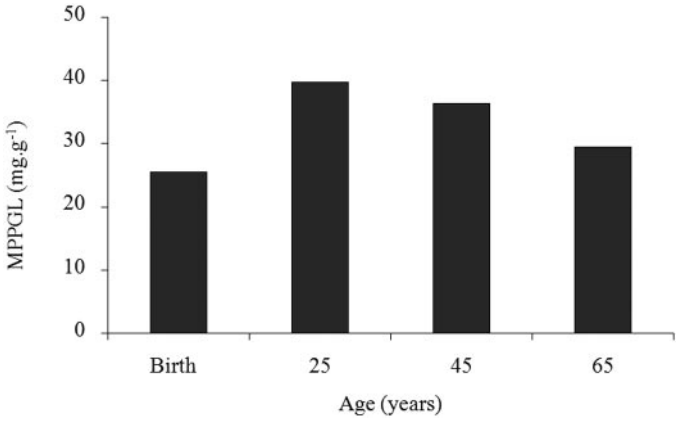

FIG. 3. Examples of age-specific values of MPPGL determined using model 4.

or microsomal (nanomoles per milligram) P450 content of fresh and frozen tissue samples, indicating that, provided care is taken before storage, samples stored at −80°C are suitable for use in the determination of MPPGL. Additional support for the use of frozen tissue as a reliable source from which to generate values of MPPGL comes from the investigation of levels of CYP420 in microsomal samples prepared from frozen tissue. We showed previously (Wilson et al., 2003) that levels of CYP420 in a subset of samples from five livers contributes less than 3% of the total CYP450, again indicating that with use of the protocols described in the current study tissue degradation is minimal.

A further experimental variable explored in the current study was the influence of different operators on the determination of MPPGL. Increasing the number of operators is advantageous in studies, such as the present one, in which the nature of the analysis makes sample processing time-consuming. As the aim of the study was to investigate interindividual variability in MPPGL, it was important to assess the impact, if any, of interoperator variability on MPPGL determination. Differences in operators affected the yield of microsomal protein. However, measurement of microsomal yield does not account for the fraction of microsomal protein lost during the centrifugation process, which can be substantial (Wilson et al., 2003). Determination of total MPPGL requires correction of microsomal yield via application of a recovery factor (1 − fraction of microsomal protein lost) (Fig. 2). Application of recovery factors for each operator should yield comparable values of MPPGL, as demonstrated in the present study. MPPGL values determined within the same laboratory by different individuals may, therefore, be compared. The effect of interlaboratory variables such as instrumentation and reagents on values of MPPGL has yet to be determined. Differences in instrumentation and reagents are also likely to produce different yields of microsomal protein. However, in theory, application of recovery factors specific to the conditions under which microsomal samples are prepared should produce similar corrected values of MPPGL across laboratories.

Collation of samples from donors aged 11 to 80 years (n = 113 adult; n = 1 pediatric) previously suggested that the relationship between human MPPGL and age decreases monotonically with age (Barter et al., 2007). Incorporation of additional samples from younger donors (n = 4 pediatric from the current study; n = 11 fetal) (Pelkonen et al., 1973) has indicated a nonmonotonic relationship between age and MPPGL with values increasing from birth to a maximum of 40 mg g^{-1} at approximately 28 years followed by a gradual decrease in older age. Values of MPPGL were approximately 36 and 31% lower in newborn and elderly (80 years) individuals than those in a 25-year-old individual (typically the age of individuals used in clinical pharmacology studies). The use of a value of MPPGL of 40 mg g^{-1}, determined for a young adult, would be expected to result in an overprediction of clearance in very young or very old patients. Therefore, MPPGL values relevant to the age of the population in which predictions are being made should be used in IVIVE.

Although the correlation between predicted and observed values of MPPGL determined using model 4 was statistically significant, the relationship between MPPGL and age only explained 10% of the overall observed variation in the data. The remaining 90% cannot be attributed wholly to experimental variability, as previous use of the repeated-measures study design (Barter et al., 2007) has demonstrated that experimental variability contributes only 20% to the overall variability in MPPGL. All investigators working in the area are, therefore, encouraged to record attributes of tissue samples to aid identification of these additional sources of variability.

In addition to further investigating the relationship between age and MPPGL, we have demonstrated that MPPGL values may be determined from frozen tissue by multiple operators. It is hoped that the provision of detailed protocols and validation studies such as those in the present study will serve to encourage wider investigation of MPPGL (including further exploration of the effect of tissue storage conditions, instrumentation, and reagents on values of MPPGL). This investigation will facilitate the identification of further covariates and examination of other factors such as ethnicity.

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