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Original Research

Utility of Orchidometric Parameters for Assessing Sexual Maturation in Male Cynomolgus Macaques (*Macaca fascicularis*)

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Testicular volume is one of several parameters that have been used in preclinical toxicology to facilitate the identification of sexually mature male cynomolgus macaques when semen evaluation is unavailable. Furthermore, testicular volume provides additional information to pathologists to aid in the interpretation of microscopic findings. Orchidometry has been proposed as a useful tool for assessing testicular volume. To assess its utility for this purpose, we used orchidometry to measure testicular volume in untreated control male cynomolgus macaques during preclinical toxicology studies. Additional parameters including age, body weight, testicular weight, serum testosterone, and testicular histology were also evaluated. Serum inhibin B and the diameter of histologic testicular sections were assessed to determine whether they might provide any additional corroborative evidence for differentiating stages of sexual maturity in males. Orchidometry was easy to use in sedated or awake macaques and, in combination with testicular histology, enabled the establishment of cut-off values by which sexually mature male cynomolgus macaques can be identified with a high degree of confidence. The relative utility of the parameters examined for discriminating sexually mature and immature males was testicular volume ≥ serum testosterone > body weight > age; for differentiation of sexually mature and peripubertal males the order was testicular volume ≥ body weight > serum testosterone > age. Testicular weight and the diameter of histologic testicular sections provided corroborative information for discriminating stages of sexual maturity. Serum inhibin B was of little value in helping to differentiate the different stages of sexual maturation evaluated in this study.

In 2011, a revised guidance for the testing of biotechnology-derived pharmaceuticals was issued by the International Conference on Harmonization. As part of this revision, the guidance specified that evaluation of the reproductive tract in sexually mature male and female NHP in studies of at least 3 mo in duration would be acceptable for assessment of potential effects on fertility when NHP are the only relevant species for toxicity testing.[10] Although this stipulation largely eliminates the need for using large numbers of animals in lengthy, expensive, and complicated mating studies in support of the reproductive toxicity evaluation of biotherapeutics, it emphasizes the importance of having reasonably reliable methods for identifying sexually mature NHP for inclusion in longer-term (longer than 3 mo) general toxicity studies. Demonstration of regularly occurring menses is a well-accepted and easily monitored endpoint for the identification of sexually mature female NHP for inclusion in toxicity studies. For males, the evaluation of ejaculates for the presence of sperm has been shown to reliably identify sexually mature male macaques.[15] However, not all animal facilities are equipped to obtain and evaluate semen samples from NHP. Therefore, the identification of a readily implemented and reasonably accurate surrogate would be helpful, particularly when faced with an unexpected need to identify sexually mature male macaques for inclusion in toxicity studies.

Histopathology has been considered the 'gold standard' for the assessment of testicular toxicity and verification of male sexual maturity in conventional toxicity studies.[21] Criteria for the histologic evaluation of testicular maturity and examination of the spermatogenic cycle in cynomolgus macaques are readily available.[6,8,13,26] But histopathology is a retrospective tool and, therefore, only confirms whether an individual animal was sexually mature at the end of the toxicity study. In addition, problems have been recognized in differentiating testicular toxicity from normal testicular development during puberty in NHP by using histology.[6,14] From the pathologist's standpoint, then, a useful surrogate marker for sexual maturation obtained prior to the beginning of treatment would be valuable for selecting animals for inclusion in studies and, on occasion, as an aid in the interpretation of testicular histopathology.

Several quantitative measures including age, body weight, testicular size (volume), serum hormone levels (particularly testosterone), sperm evaluation, and conception rate have been evaluated as in vivo markers of sexual maturity in male cynomolgus macaques.[4,8,12,14,15,18,19,26,27] In most reports evaluating the utility of testicular volume, calipers have been used to measure the testes, and volume was then calculated by using a formula for the volume of an ellipsoid.[12,19,20,23,26,27] One group evaluated testicular

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volume by using an orchidometer to identify sexually mature male NHP and found that this tool appeared to offer a reasonable alternative to the use of calipers.[13] Advantages of orchidometry include the low cost of the apparatus, the speed with which measurements can be obtained, and the ease with which the procedures can be incorporated into the conduct of a toxicity study.

We were interested in further evaluating the practical utility of orchidometry for identifying sexually mature male macaques in our study population and for informing the study pathologist of the likely maturity status of animals prior to treatment. Therefore, we prospectively compared orchidometry and other in vivo (age, body weight, serum testosterone) and ex vivo (testicular weight and diameter) markers for sexual maturity with testicular histology in male cynomolgus macaques. As a component of this work, we also investigated the utility of the circulating testicular hormone, inhibin B, to determine whether it might provide any adjunctive information to identifying sexually mature male macaques.

Materials and Methods

Animals and husbandry. The animals used in these investigations were purpose-bred Mauritian male cynomolgus macaques. All animals were randomly assigned as untreated vehicle control animals ($n = 99$) in preclinical toxicology studies conducted at Pfizer Worldwide Research and Development facilities (Groton, CT). The macaques were housed individually or in same-sex pairs in stainless steel cages and acclimated to the facility for a minimum of 30 d prior to study initiation. Environmental conditions maintained in the study rooms included: room temperature of 66 °F to 77 °F, humidity of 30% to 70%, and a 12:12-h light:dark cycle. Macaques were fed pelleted food (Primate Diet 5K91, PMI Feeds, St Louis, MO) supplemented with vegetables and fruit and had access to municipal drinking water further purified by reverse osmosis. The animals were monitored daily for health and food consumption, and body weights were assessed before, at varying times during, and at the end of each study. To facilitate handling, macaques were fitted with collars while in quarantine. Collared animals were acclimated to weekly or biweekly pole-and-chair restraint over 4 to 6 wk. Evaluations to ensure appropriate acclimation were performed by the veterinary staff before the animals were released for use on study. All procedures involving animals were conducted under an animal use protocol approved by Pfizer's IACUC and were in compliance with all applicable federal regulations and the *Guide for Care and Use of Laboratory Animals*.[9]

Orchidometry. Orchidometric assessment of testicular volume was conducted on all animals by using only chair restraint prior to study start during protocol-directed procedures. At the end of each study, orchidometry was conducted either in awake macaques with chair restraint during protocol-directed procedures (approximately 1/3 of animals) or after sedation of macaques with ketamine (20 mg/kg IM) prior to necropsy (approximately 2/3 of the animals). Testicular volume was measured by using an orchidometer (Test Size Orchidometer, model RA-125 [1 to 25 mL] or RA-125 Plus [1 to 35 mL], Accurate Surgical and Scientific Company, Westbury, NY). The right and left sides of the scrotum were palpated separately to isolate each testicle for comparison with the ellipsoidal reference beads (individual beads in increments of 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25, 30, and 35 mL). The populations of reference beads on both models of orchidometer were identical, with the addition of 30- and 35-mL ellipsoids on the RA-125 Plus model. For each testicle examined, the closest match during comparison with the orchidometer was used as the individual testicular volume. All measurements were obtained by laboratory personnel trained in the use of the orchidometer.

Serum hormones. Serum was obtained from each macaque (restrained in chairs or sedated) by venipuncture of the femoral vein in the morning prior to necropsy, to minimize effects of diurnal variation on testosterone.[28] Individual samples for testosterone and inhibin B were frozen in aliquots at –20 °C until assayed. Serum testosterone was assayed in a 15-µL sample on an automated immunoassay system (Advia Centaur, Siemens Medical Solutions, Malvern, PA) using direct chemiluminescence (catalog no. 7207660; Siemens AG, Munich, Germany) with an assay range of 10 to 1500 ng/dL. Inhibin B was measured by using 50-µL serum per well in an ACTIVE Inhibin B Gen II ELISA assay kit (reference no. A81301, Diagnostic Systems Laboratories, Beckman–Coulter, Brea, CA), with an assay range of 5 to 1000 pg/mL. All assays were performed according to the manufacturer's instructions.

Collection and examination of testes and epididymides. At the end of each study, animals were sedated (20 mg/kg ketamine hydrochloride IM), anesthetized (100 mg/kg sodium pentobarbital IV via the cephalic or saphenous vein) to a surgical plane, and then exsanguinated. Testes and epididymides were removed from each macaque at necropsy, and each epididymis was separated from the testis. Paired or individual testicular weights were obtained (as directed by study protocol); when individual weights were obtained, they were combined for comparisons. The diameter of each intact testis was determined by using calipers (Absolute Digimatic Caliper, Mitutoyo, Kawasaki, Japan) at approximately the midpoint along the longitudinal axis, the diameter (mm) was recorded, and the average diameter for each animal was used for comparisons; in one case, when both testes were not available, no measurement was recorded for the animal. Testes and epididymides were preserved in modified Davidson solution (catalog no. 64133-SP, Electron Microscopy Sciences, Hatfield, PA). Each testis was trimmed according to laboratory standards to provide a single transverse section at the level of the rete testis. Epididymides were halved and then trimmed longitudinally to facilitate examination of the head, body, and tail. Testes and epididymides were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light-microscopic examination.

During microscopic review, macaques were classified by the histologic appearance of the testes and epididymides by using a modification of previously defined criteria as immature, peripubertal (early adolescent and adolescent), or mature (early adult and adult).[13] Briefly, immature animals exhibited early spermatogenesis with an absence of spermiogenesis in the testes accompanied by empty epididymal ducts. In peripubertal macaques, spermatogenesis was present, with limited spermiogenesis in scattered tubules and little or no epididymal spermatozoa. Mature animals exhibited spermatogenesis with spermiogenesis in most or all seminiferous tubules and the presence of moderate to large numbers of spermatozoa and rare nucleated cells in epididymal ducts.[13] After microscopic evaluation, the diameter of each transverse histologic section of testis was measured (in mm) by using calipers (at the largest cross-sectional point of the section), and the average testicular diameter for each animal was determined.

Statistical analysis. Measurements of age (y), body weight (kg), testicular diameter (mm), testicular weight (g), testicular volume

Table 1. Population parameters relative to histologically defined sexual maturation of male cynomolgus macaques

	Immature			Peripubertal			Mature			
	Mean ± 1 SD	Range	n	Mean ± 1 SD	Range	n	Mean ± 1 SD	Range	n	Total n
Age (y)	3.2 ± 0.3	2.8 to 3.8	26	3.3 ± 0.4	3.0 to 4.2	11	4.5 ± 1.0[c,d]	3.0 to 6.8	62	99
Body weight (kg)	3.5 ± 0.4	3.0 to 4.6	26	3. 6 ± 0.5	2.9 to 4.0	11	5.5 ± 1.3[c,d]	3.5 to 9.5	62	99
Testicular diameter (mm)[a]	9.6 ± 1.0	7.2 to 11.8	26	13.9 ± 2.8[c]	11.0 to 19.6	11	21.0 ± 2.9[c,d]	14.7 to 27.6	61	98
Testicular weight (g)[b]	2.1 ± 0.5	1.3 to 3.2	24	7.7 ± 5.3	3.6 to 19.9	10	33.4 ± 11.7[c,d]	12.0 to 57.6	55	89
Testicular volume (mL)[b]	4 ± 1	2 to 6	22	12 ± 7[c]	5 to 24	10	37 ± 11[c,d]	12 to 70	51	83
Testosterone (ng/dL)	51 ± 36	<20 to 160	22	156 ± 116	62 to 438	10	538 ± 356[c,d]	112 to >1500	55	87
Inhibin B (pg/mL)	963 ± 78	706 to >1000	21	921 ± 134	594 to >1000	10	827 ± 170[c]	458 to >1000	52	83

[a]Average of 2 testes measured on histology slides.
[b]Both testes combined.
[c]$P < 0.05$ compared with values from immature animals.
[d]$P < 0.05$ compared with values from peripubertal animals.

(mL), and the serum hormones testosterone (ng/dL) and inhibin B (pg/mL) were analyzed by using SAS version 9.4 (SAS Institute, Cary, NC). Pearson correlation coefficients were calculated to assess the strength of relationships among these different parameters.

The study macaques were classified into immature, peripubertal, and mature categories. For each parameter, pairwise comparisons between mature and peripubertal animals, between mature and immature animals, and between peripubertal and immature animals were done by using *t* tests with Tukey multiple comparison adjustment. Tests were conducted at the 5% significance level.

Cutoff values for each of the parameters were calculated for the purpose of determining maturity of macaques. By using data from peripubertal animals, upper tolerance limits for all parameters, except inhibin B, were calculated corresponding to a 90% confidence that 95% of peripubertal animals have values that do not exceed the upper limit. Because inhibin B has an inverse relationship with maturity, the lower tolerance limit was calculated such that there is 90% confidence that 95% of peripubertal animals have inhibin B values no less than the lower limit. Similar calculations using data for immature macaques were conducted to obtain the tolerance limits for differentiating immature from mature animals.

Results

A total of 99 control cynomolgus macaques from 44 preclinical safety studies were available prospectively for examination. According to histologic examination of the testes and epididymides at the end of each study, 26 were classified as immature, 11 as peripubertal, and 62 as mature. Some data points were unavailable for individual animals due to insufficient samples, errors in data acquisition or recording, or study requirements prohibiting their collection. Table 1 summarizes the parameters examined for animals at each stage of maturity, including age, body weight, testicular weight, testicular volume, average histologic testicular diameter, and serum testosterone and inhibin B levels. Average testicular diameter was slightly smaller for measurements obtained from histologic sections (17.09 mm) compared with those measured at necropsy (20.09 mm). However, these 2 parameters showed high correlation (r = 0.92, $P \leq 0.0001$, $n = 86$; data not shown), and only the results for histologic diameter are presented in this report. As we expected, age, body weight, combined testicular weight, combined testicular volume, testosterone levels, and average diameter of the testicular histology sections generally increased from immature to mature groups (Figure 1). As animal age increased, a trend toward decreased inhibin B values was noted, but there was a high degree of overlap in values across all maturity grades (Figure 1).

Correlations among the various tested parameters (Table 2) were generally highly statistically significant ($P < 0.0001$), with the exception of inhibin B, which bore a relationship only with testicular diameter. In particular, we saw strong relationships among body weight, testicular weight, and testicular volume as well as good correlation between testicular volume and testicular histology (Figure 2).

Using data from immature and peripubertal animals, we developed a table of cut-off values (Table 3) to help guide the identification of mature male macaques and to compare the utility (sensitivity) of the various parameters assessed in our population. The tolerance limits were set to provide 90% confidence that 95% of animals selected, according to the cut-off values, would be sexually mature. For all parameters except inhibin B, the cut-off values were based on the upper limit of tolerance observed in peripubertal or immature animals. For inhibin B, the cutoff was based on the lower limit of tolerance in peripubertal or immature animals, because inhibin B levels bore an inverse relationship with maturity.

According to comparison of the cutoff values and the histologically assigned maturity grade for each animal (Table 3), orchidometric testicular volume and serum testosterone were the most useful of the evaluated in vivo parameters for discriminating mature from immature male cynomolgus macaques, followed by body weight, age, and inhibin B. For discrimination of mature from peripubertal animals, testicular volume proved to be equal to or slightly better than body weight, followed by testosterone, age, and inhibin B. Of the ex vivo parameters examined, testicular weight and the mean diameter of the histologic sections of testes both clearly discriminated all mature and immature male macaques. Testicular weight was a better predictor than was testicular diameter from histologic sections (82% compared with 49%, respectively) for differentiating mature and peripubertal males. Of the 10 peripubertal animals with testicular volume measurements, 2 had testicular volumes of 20 to 24 mL, as did 11 of our mature animals. To examine the effects of using different cut-off values for combined testicular volume (20, 24, or 28 mL) to

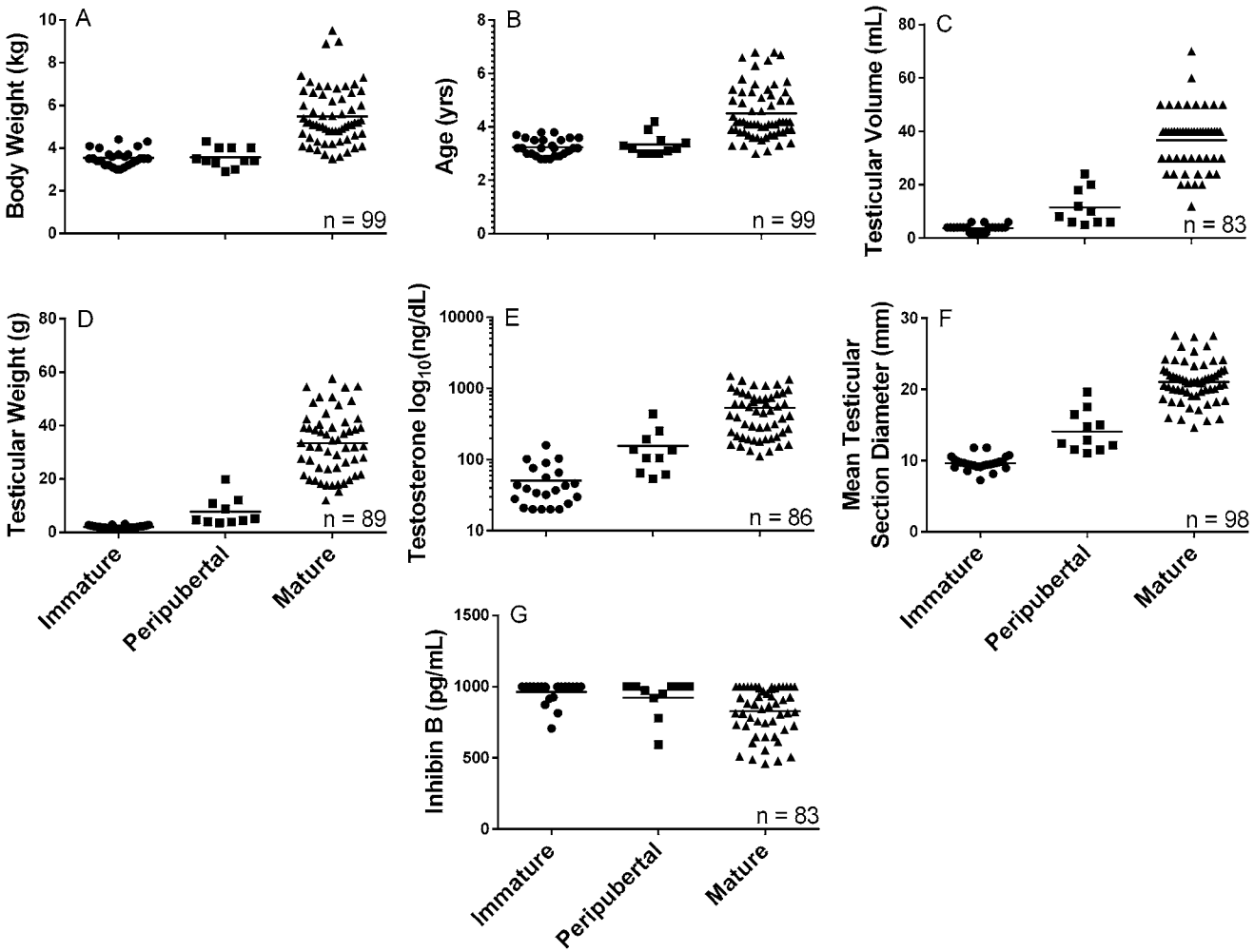

Figure 1. Relationships between histologically defined sexual maturity status (immature, peripubertal, or mature) and various other parameters of vehicle-treated male cynomolgus macaques in toxicology studies. Horizontal lines represent group mean values. The number at the lower right of each panel represents the total number of animals represented as data points. (A) Body weight. (B) Age. (C) Combined testicular volume. (D) Combined testicular weight. (E) Serum testosterone. (F) Average diameter of testicular histologic sections. (G) Serum inhibin B. For serum hormones, values above or below the linear range of the assay were adjusted to the upper or lower limit of quantitation for calculations and display.

identify mature male macaques, we maintained a 90% confidence level and determined the corresponding coverage probability, specificity, and sensitivity for the discrimination of mature and peripubertal monkeys by using our study data (Table 4).

Discussion

Orchidometry discriminated sexually mature and immature male cynomolgus macaques in vivo with a high degree of confidence by using population-based cut-off values anchored against testicular histology. As expected, discrimination of adult and peripubertal males with similar confidence was difficult due to the variability in testicular volume in peripubertal animals. Of the various in vivo parameters we evaluated for selection of mature male animals, orchidometry had high utility for discriminating this population from both immature (orchidometry ≥ serum testosterone > body weight > age) and peripubertal (orchidometry ≥ body weight > serum testosterone > age) animals. Testicular weight and testicular section diameter, both measured ex vivo, also were useful in discriminating mature animals from those that were immature or peripubertal. Inhibin B had no value for discriminating sexually mature animals from those at earlier stages of sexual development.

Several in vivo parameters have been used to identify sexually mature male macaques, including body weight greater than 4.5 kg, age greater than or equal to 4 to 5 y, combined testicular volume greater than 10 mL, serum testosterone greater than 15 to 20 nmol/L, and proof of sperm in an ejaculate.[30] But with the exception of an ejaculate sample containing sperm, none of these criteria have proven entirely trustworthy. We initiated the current study to further examine the utility of orchidometry as a simple, easily implemented, and reasonably accurate in vivo method for identifying mature male macaques for use in preclinical toxicology studies. This inquiry was driven by an increased demand for inclusion of mature male monkeys in studies supporting clinical programs in biotherapeutics as well as a sporadic need for in-life data to assist veterinary pathologists in the interpretation of testicular morphology at the end of preclinical toxicology studies.

In the past, testicular volume in cynomolgus macaques has typically been determined by taking testicular measurements with

Table 2. Correlations among parameters

	Age (y)	Body weight (kg)	Testicular diameter (mm)[a]	Testicular weight (g)[b]	Testicular volume (mL)[b]	Testosterone (ng/dL)	Inhibin B (pg/mL)
Age (y)	1.00	0.85	0.59	0.77	0.79	0.53	−0.17
P		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.13
n	99	99	98	89	83	87	83
Body weight (kg)		1.00	0.68	0.85	0.83	0.58	−0.18
P			<0.0001	<0.0001	<0.0001	<0.0001	0.11
n		99	98	89	83	87	83
Testicular diameter (mm)[a]			1.00	0.85	0.89	0.66	−0.23
P				<0.0001	<0.0001	<0.0001	0.04
n			98	88	83	86	82
Testicular weight (g)[b]				1.00	0.94	0.70	−0.12
P					<0.0001	<0.0001	0.30
n				89	76	80	77
Testicular volume (mL)[b]					1.00	0.65	−0.09
P						<0.0001	0.44
n					83	76	74
Testosterone (ng/dL)						1.00	−0.06
P							0.59
n						87	83
Inhibin B (pg/mL)							1.00
P							
n							83

[a]Average of 2 testes as measured on histology slides.
[b]Both testes combined.

calipers and calculating testicular volume by using a formula for an ellipsoid.[12,19,20,23,26,27] However, the use of calipers is somewhat involved, given that sedation or anesthesia typically are required and that considerable inter- and intraobserver variability has been reported, sometimes leading to unreliability of the measurements.[26,30] In addition, ultrasonography has been used successfully to evaluate testicular volume in cynomolgus macaques,[15] but this methodology requires sedation of the animals as well as the availability of ultrasound equipment and personnel trained in its use. One group of researchers has reported preliminary results suggesting the use of orchidometry as an effective means for identifying mature male macaques.[13] Expanding this previous work, we sought to obtain additional support for the use of this tool to select sexually mature males for use in toxicology studies. Our current study demonstrates, in a larger cohort of animals than used previously,[13] the utility of orchidometry for estimating testicular volume and its value for identifying sexually mature male cynomolgus macaques.

We found that the categorical measurements made with the orchidometer were obtained easily with or without chemical restraint and were sufficiently informative to identify a large proportion of mature male macaques. In this study, the use of sedation was generally a matter of convenience for obtaining measurements from animals prior to necropsy. However, measurements were satisfactorily obtained by using only chair restraint for all animals before study and in about 1/3 of macaques at end of study. In those animals in which a change in maturation status was not apparent over time, the prestudy and end-of-study orchidometry measurements were generally very similar regardless of the method of restraint used (data not shown). In our experience, orchidometry has a number of advantages, including minimal investment, minimal requirements for restraint, ease of training and use by study and veterinary personnel, ready incorporation into and minimal disruption of study activities, and high value for classifying animals by likely maturity status. Furthermore, the categorical nature of the measurements obtained with orchidometry, although less precise than those provided by calipers, are sufficiently robust in the hands of experienced personnel and across multiple observers to classify male macaques as sexually immature, peripubertal, or mature animals. From our perspective, these 3 categories are sufficient to provide guidance about the likely maturation state of individual animals and to support decisions regarding their selection for use in preclinical toxicology studies.

A combined testicular volume of 10 to 25 mL has been recommended as one criterion for identifying sexually mature male cynomolgus macaques.[12,13,19,30] We and others[20] have observed that testicular volume in peripubertal male macaques occasionally

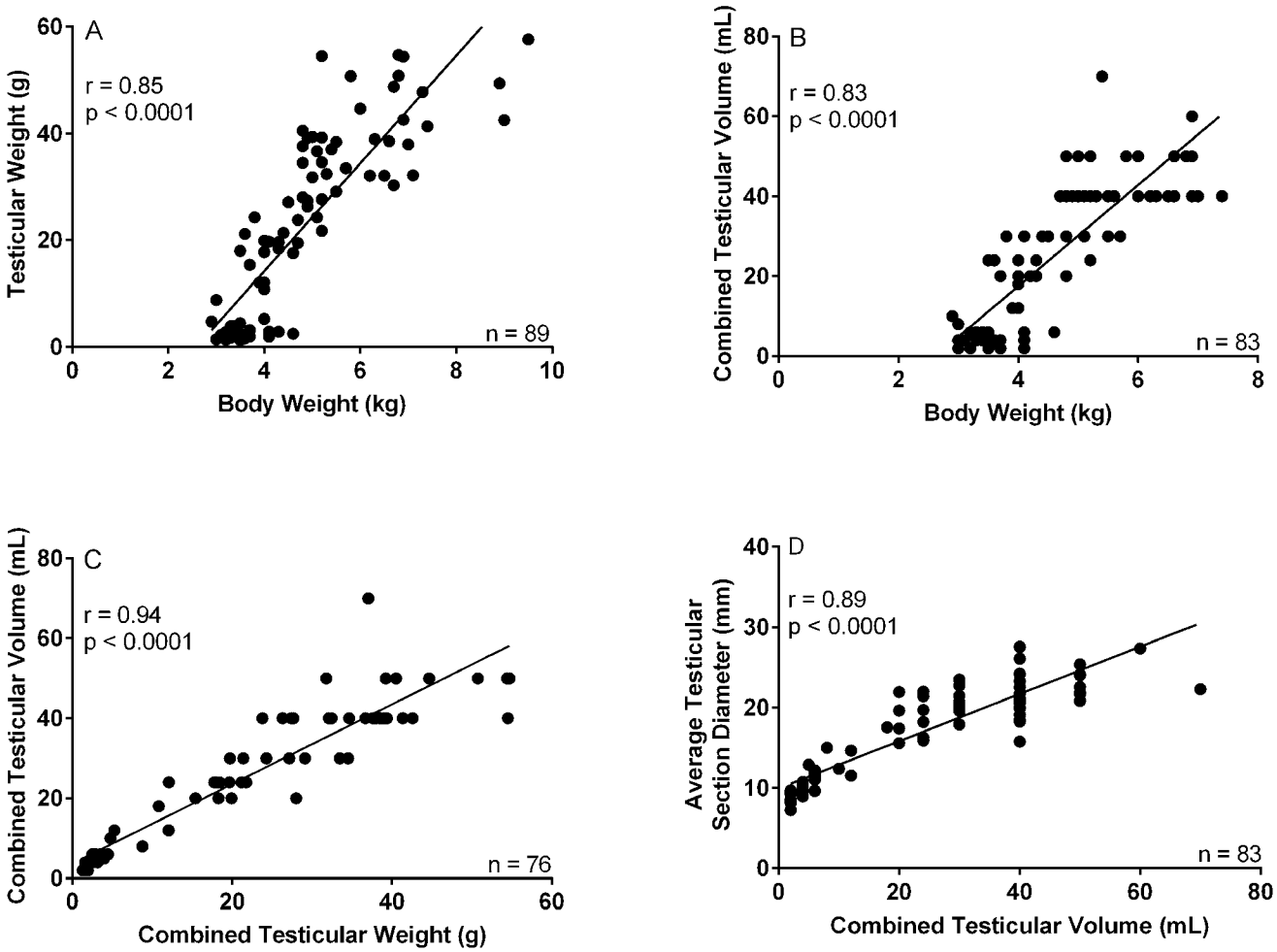

Figure 2. Relationships among various parameters in vehicle-treated male cynomolgus macaques used in toxicity studies, with linear regression curves indicated. r, correlation coefficient; *n*, number of animals used to evaluate the relationship. (A) Body weight and combined testicular weight. (B) Terminal body weight and combined testicular volume. (C) Combined testicular weight and combined testicular volume. (D) Combined testicular volume and average diameter of testicular histologic sections.

exceeds 20 mL, and our data and modeling indicated that a cutoff of 28 mL is highly effective for identifying mature animals. From a practical standpoint, the use of a cutoff of 28 mL would be appropriate when screening animals for inclusion in a study where there are concerns of test-article–related testicular effects. In that case, a relatively high level of certainty is desirable, and this level of stringency would provide the least likelihood of encountering confounding histologic changes related to incomplete maturation. When possible, demonstration of sperm in the ejaculate is warranted for pivotal assessments of male reproductive toxicity to verify that mature animals had been selected.[12,15] When there are few concerns regarding toxicologic effects on the testes, however, particularly when semen evaluation is unavailable, we might elect to use the less-stringent cutoff of greater than or equal to 20 mL to screen for mature male macaques. This revision would expand the pool of animals available for study, yet still provide us with 90% confidence that 80% of the animals selected would be mature (Table 4), recognizing that occasional peripubertal animals might be selected.

It may be worth bearing in mind that the current work was designed to evaluate orchidometry against the gold standard of histology for identifying mature animals for use in toxicity studies. For other purposes, such as breeding, there is limited information on the use of orchidometry for assessment or prediction of reproductive performance. One author considered testicular volume of greater than or equal to 25 mL to be required for a high likelihood for reproductive success but found that about 10% of animals meeting this criterion still failed to produce offspring.[19] Therefore, the utility of orchidometry for the prospective evaluation of reproductive performance requires further evaluation.

With regard to other in vivo markers that we examined, our results were similar to findings published elsewhere. Body weight, with a cut-off of 4.7 kg, was a reasonably good surrogate for selecting sexually mature male macaques in our population and offered roughly similar utility as testicular volume. This cut-off for selection is similar to previously published recommendations of 4.5 to 5.3 kg supported by histology.[8,13-15,26,30] Testosterone levels increase with maturity but were a less robust marker for distinguishing mature from peripubertal animals than were testicular volume and body weight. Although testosterone levels of immature and peripubertal animals were uniformly below 450 ng/dL (15.6 nmol/L) and only a single value in these 2 groups

Table 3. Utility of cutoffs for identification of sexually mature male cynomolgus macaques

	Discrimination from immature		Discrimination from peripubertal	
	Cutoff value	Mature animals exceeding cutoff	Cutoff value	Mature animals exceeding cutoff
Testicular weight (g)[a]	3.1	55/55 (100%)	21.0	45/55 (82%)
Testicular volume (mL)[a]	6	51/51 (100%)	28	40/51 (78%)
Testicular diameter (mm)[b]	12.0	61/61 (100%)	21	30/61 (49%)
Body weight (kg)	4.3	49/62 (79%)	4.7	44/62 (71%)
Age (y)	4.0	40/62 (65%)	4.3	26/62 (42%)
Testosterone (ng/dL)	125	54/55 (98%)	450	28/55 (51%)
Inhibin B (pg/mL)	790	29/52 (56%)	580	16/52 (31%)

Data are given as no. of macaques meeting the criterion/no. evaluated in total (% of population)
[a]Both testes combined
[b]Average of 2 testes as measured on histology slides

Table 4. Sensitivity and specificity for discrimination of mature and peripubertal macaques by using different cutoffs for testicular volume

Cutoff (mL)[a]	Confidence level (%)	Coverage probability	Sensitivity	Specificity
28	90	0.95	0.78	1.00
24	90	0.88	0.90	0.90
20	90	0.80	0.98	0.80

[a]Based on upper limit of tolerance for peripubertal male cynomolgus macaques.

exceeded 300 ng/dL (10.4 nmol/L; data not shown), only half of the histologically mature animals had testosterone levels above the cutoff for peripubertal animals (Table 3). Age was the least useful parameter for selection of mature animals in our cohort, and this finding is consistent with the wide age range (3.5 to 7 y) across which male cynomolgus macaques attain sexual maturity.[4,8,14,15,26] Several authors have shown that, in general, male cynomolgus macaques should be older than 5 y to have reasonable confidence that they are sexually mature.[4,14,26] In our study cohort, the youngest mature male macaque was 3.0 y old and the oldest peripubertal animal was 4.2 y of age, but only 20 of 62 mature male macaques were 5 y or older (data not shown). For our population of animals, the use of conventional cut-off values from the literature for testosterone or age would have proven overly conservative for selecting mature male cynomolgus macaques, leading to a much smaller pool of animals available for study.

As expected, we found that the 2 ex vivo measures examined, combined testicular weight and the average diameter of testicular sections on slides, both correlated well with sexual maturity. Strong correlations have previously been shown for maturity grade (based on histology) compared with testicular weight and for testicular weight compared with volume.[8,13,20]

Because testicular diameter is an important variable in determining testicular volume,[30] it seemed reasonable that diameter of the histologic section might be useful to the pathologist if microscopic findings were to raise questions about maturity and when appropriate in vivo data were unavailable to help with this determination. One previous study used histologic sections to demonstrate perspective on the differences in testicular size at different stages of maturity.[6] Otherwise, few objective data support the consideration of testicular diameter as an aid in discriminating sexual maturity, and there are some caveats in considering such a proposal. First, many factors during slide preparation may affect the final size and shape of a tissue section and it is not an ideal specimen for drawing definitive conclusions on maturity. In addition, sections must be obtained consistently from all animals to have any validity for comparisons; in our laboratory, the rete testis, slightly dorsal to the center of the longitudinal axis, is used as a landmark for consistent trimming of transverse sections for examination. With attention to these considerations, we were able to achieve high correlation between the diameter of testicular histology sections with testicular weight and testicular volume. In our population, the cutoff value for average histologic testicular diameter (21 mm) clearly distinguished animals that had not yet reached sexual maturity (Table 1). However, testes of only 49% of our mature male macaques exceeded this value; this result may reflect both the variability in testicular size within this group as well as inherent inconsistencies in sampling and histologic processing. Therefore, measurements of testicular diameter in histologic sections, by itself, appears to be of little value for confidently classifying male cynomolgus macaques s as sexually mature.

Determination of histologic diameter of the testis may still have utility as part of a weight-of-evidence approach to evaluation of sexual maturity. Spermatogenesis is a homogeneous process in the maturing testis,[5] although peripubertal development may not be uniform in all tubules and its histologic appearance may mimic a toxicologically induced change;[6,14,29] our own experience confirms these observations. In cases where the pathologist is faced with equivocal histologic findings suggestive of either toxicity or pubertal maturation, testicular diameter in combination with other data may help provide an objective basis for discrimination. For instance, if the combined testicular weight exceeds 20 g (the highest weight observed in our peripubertal animals) and average testicular cross-sectional diameter on the slide exceeds 21 mm, the pathologist can be reasonably sure that the animal was likely to be sexually mature at the end of the study. In such cases, the presence of histologic changes in the spermatogenic epithelium such as degeneration, luminal exfoliation, or cellular depletion, especially occurring with a relationship to increasing dose, is more likely to be due to toxicity than to normal peripubertal development. However, it is expected that cases will still arise when testicular weight, average cross-sectional diameter, or testicular volume falls into a 'gray zone' between the lower end of the mature range and the upper end of the peripubertal range, resulting in uncertainty around the histologic discrimination of peripubertal from toxicologically induced changes.

Inhibin B is a glycoprotein hormone produced principally by Sertoli cells in the testes and involved in the regulation of follicle-stimulating hormone secretion.[22] In human and rhesus macaque

males, circulating levels of inhibin B are high in infants, low in juveniles, and rise during adolescence to adult levels.[1,3,16,17] After adolescence, inhibin B levels tend to decrease with normal aging.[2,25] In general, circulating levels of inhibin B reflect both the size of the healthy Sertoli cell population and normal spermatogenic function.[2,24] The patterns of circulating inhibin B levels in male cynomolgus macaques are qualitatively similar from birth to maturity[11] and, at least in adults, are reportedly quantitatively similar to those of rhesus macaques,[7] but little data for this hormone during maturation is otherwise available for cynomolgus animals. Therefore, we measured inhibin B levels to determine whether this hormone might provide additional information for discriminating male cynomolgus macaques at different stages of sexual maturity. Although levels in mature males were lower on a group-mean basis relative to immature males in our cohort, inhibin B was not significantly correlated with age, body weight, testosterone, testicular volume, or testicular weight in our study animals. In all groups, a large number of animals had serum values at or above the upper limit of quantitation and, because of this overlap, inhibin B provided no additional value for stratifying animals for sexual maturity. As noted earlier, serum inhibin B levels typically increase as animals mature from juvenile to adult, but our data demonstrated high levels of circulating inhibin B in histologically immature monkeys. This result was somewhat unexpected but might reflect (at least in part) the older ages of our immature animals (Table 1) compared with those of the previously described juveniles (1.5 to 3.5 y)[11] and suggests that increased inhibin secretion by Sertoli cells may precede morphologic evidence of testicular maturation during early puberty in cynomolgus macaques.

The literature contains a fairly wide range of values for the markers we examined in the current study. In part, this variability is likely due to the relatively small number of animals evaluated in earlier studies as well as interindividual variation in the onset of and progression through puberty, but the genetic background of the animals used in the various reports is not always clear and may have played a role. The influence of genetics was clearly demonstrated by 2 authors who examined differences changes in body weight and testicular maturation in a large cohort and showed that body growth and testicular maturation occurred earlier in animals of Mauritian origin than in animals of Asian mainland origin.[15] Therefore, although the findings from the current study are likely to be helpful in a general sense, their use with animals of Asian mainland descent requires verification.

In conclusion, our findings demonstrate that orchidometry is a useful and easily implemented method for screening male cynomolgus macaques for sexual maturity, particularly when more definitive methods for assessing maturity, such as semen evaluation, are not readily available. In addition, we found that measurement of inhibin B did not contribute additional information for discriminating mature from sexually immature males in the population of cynomolgus macaques available for our preclinical studies.

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