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Clinical Chemistry Reference Intervals for C57BL/6J, C57BL/6N, and C3HeB/FeJ Mice (*Mus musculus*)

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Although various mouse inbred strains are widely used to investigate disease mechanisms and to establish new therapeutic strategies, sex-specific reference intervals for laboratory diagnostic analytes that are generated from large numbers of animals have been unavailable. In this retrospective study, we screened data from more than 12,000 mice phenotyped in the German Mouse Clinic from January 2006 through June 2014 and selected animals with the genetic background of C57BL/6J, C57BL/6N, or C3HeB/FeJ. In addition, we distinguished between the C57BL/6NTac substrain and C57BL/6N mice received from other vendors. The corresponding data sets of electrolytes (sodium, potassium, calcium, chloride, inorganic phosphate), lipids (cholesterol, triglyceride), and enzyme activities (ALT, AST, ALP, α-amylase) and urea, albumin, and total protein levels were analyzed. Significant effects of age and sex on these analytes were identified, and strain- or substrain- and sex-specific reference intervals for 90- to 135-d-old mice were calculated. In addition, we include an overview of the literature that reports clinical chemistry values for wild-type mice of different strains. Our results support researchers interpreting clinical chemistry values from various mouse mutants and corresponding wild-type controls based on the examined strains and substrains.

Abbreviation: GMC, German Mouse Clinic

Mice are widely used as model organisms to investigate biologic mechanisms in health and disease but also to establish new diagnostic and therapeutic strategies. The numbers of animals used are continuously rising (http://www.understanding-animalresearch.org.uk/the-animals/numbers-of-animals), and most of them are needed for pharmacology, oncology, toxicology, drug safety, forward and reverse genetic, and other studies. Such research typically involves the use of inbred strains, which are generated through at least 20 generations of brother×sister mating.[3,28,29] The uniform genetic background of inbred mice improves standardization and helps researchers worldwide to compare their results with sufficient reproducibility, thereby minimizing the repetition of experiments.[31] Widely used inbred mouse lines include several strains of C57BL/6 and C3H origin; these mice are available from different internationally operating vendors.

Within these strains, various substrains with specific genetic and phenotypic characteristics are available. For example, C57BL/6 was established in 1921 at the Bussey Institute for Research in Applied Biology and, at F24 in 1948, traveled to the Jackson Laboratory (C57BL/6J). In 1951 at F32, this strain was passed to the NIH, and continued isolated breeding resulted in a new substrain, C57BL/6N. The journey continued to various vendors, who currently offer several different but related mouse substrains, such as the C57BL/6NTac line, which was established in 1991 at F151. During the 220 generations of evolution, the backgrounds of the C57BL/6J and C57BL/6N substrains separated from each other. Therefore not only strains but also substrains can demonstrate genetic discrepancies[28] that account for differences in findings between laboratories,[8,19,23,40] structural phenotypes,[30] behavior,[39,41,43] and responses to different stimuli.[6,10,22]

In addition to strain- and substrain-specific features, sex- and age-dependent differences in phenotypic traits have been described.[2,12,27,47] Furthermore, significant effects of housing conditions and experimental procedures have been reported for many clinical chemistry plasma analytes in mice.[7,11,23] The high number of influencing factors makes it difficult to define universally valid 'normal values'. Although reference intervals defining normal levels have been reported for various analytes[4,35,38,42] and although some vendors provide such data for specific experimental animals, a detailed analysis of literature demonstrated the lack of sufficient data from large cohorts adjusted for sex, age, and strain or substrain. Therefore, few established reliable reference intervals are available. The purpose of the current study was to summarize sex-specific clinical chemistry reference intervals for healthy C57BL/6J, C57BL/6N, C57BL/6NTac, and C3HeB/FeJ mice, according to data collected during standard clinical-chemistry phenotyping in the German Mouse Clinic (GMC).[14] In addition, we present an overview of published studies that present reference data for plasma clinical chemistry analytes.

Materials and Methods

Animals. For this large retrospective study, data from more than 12,000 healthy wild-type mice investigated at the GMC

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Table 1. Characteristics of the mice included in the calculation of reference interval

	C57BL/6NTac	C57BL/6N	C57BL/6J	C3HeB/FeJ	Overall
Mice screened					
No. of male mice	684	116	496	150	1446
No. of female mice	714	148	463	161	1486
Total no. of mice	1398	264	959	311	2932
No. of cohorts	92	12	43	14	161
No. of mice (male/female) per cohort	1–25/1–24	0–15/9–26	5–24/0–19	5–16/5–16	1–25/1–26
Mice included (that is, 90–135 d old)					
No. of male mice	674	109	445	76	1304
No. of female mice	713	113	453	84	1363
Total no. of mice	1387	222	898	160	2667
No. of fresh/frozen samples	1193/194	222/0	790/108	100/60	2305/362
Number of cohorts	92	10	41	7	150
No. of mice (male/female) per cohort	1–25/1–24	8–15/9–15	5–19/6–19	4–15/4–15	1–25/1–24
No. of cohorts from source					
German Mouse Clinic	3	0	4	3	10
Helmholtz Zentrum Muenchen (no. of colonies represented)	83 (1)	0	9 (6)	3 (1)	95
Commercial vendors (no. of colonies represented)	6 (2)	0	2 (1)	0	8
Other scientific institute (no. of colonies represented)	0	10 (8)	26 (16)	1 (1)	37

from January 2006 through June 2014 were screened by using the local animal and facility management system.[26] Only mice that had undergone standardized systematic primary phenotyping in the GMC[13,15,16,17] were selected to create large cohorts of mice belonging to the strains or substrains C57BL/6J, C57BL/6N, C57BL/6NTac, and C3HeB/FeJ for data analysis. The mice either belonged to cohorts of inbred mice directly purchased by the vendor, were wild-type controls from inhouse expansion breeding for the International Mouse Phenotyping Consortium project (www. mousephenotype.org), or belonged to control groups of mutant lines bred on the respective genetic background and thus comprised wild-type littermates of mutant animals. Littermates from mutant lines were considered comparable to wild-type mice of the inbred strain and were included in the study population when the mutation had been backcrossed at least 7 times to the defined inbred background strain and animals had (according to the collaborators' information) a homozygous wild-type genotype for the gene carrying the mutation in mutants. Each cohort of mice used for standardized phenotyping usually consisted of similar numbers of male and female controls and mutant mice of similar age, not more than 3wk apart from each other.

First we excluded from the data set exported from the database all mice on hybrid genetic backgrounds or other inbred backgrounds, including those mice that had been backcrossed fewer than 7 times to the defined inbred background strain before submission to the GMC. We excluded additional cohorts for which no clinical chemistry data were available due to technical reasons. In addition, data of 12 mice were eliminated, given that several findings during the phenotyping screens comprising final pathology suggested that they were unhealthy at the time of testing.

After this curation, a total of 2947 mice belonging to the selected strains remained for inclusion in the study. The C57BL/6J mice and C3HeB/FeJ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME), and the C57BL/6NTac mice were from Taconic (Hudson, NY). The group labeled C57BL/6N contains cohorts of mice on the C57BL/6N background that were originally obtained from various independent providers except Taconic. In cases of mutant line littermates, expansion of the breeding colony and breeding of the cohort used for phenotyping was done in the facilities of collaboration partners. All cohorts of mice used for the standardized GMC phenotyping, which were not bred in the GMC, were imported into the GMC and acclimated to standard housing conditions 2 wk before the start of the phenotyping screen and, depending on the battery of tests applied, 10 to 13 wk before sample collection for clinical chemistry analyses. Detailed information on the number of subcohorts and different origins of mice are given for each strain or substrain in Table 1.

Housing, blood collection, and clinical chemistry. Mice were housed in groups of 1 to 5 animals in IVC with unrestricted access to standard mouse chow (no. 1314, Altromin, Lage, Germany) and water according to standard GMC housing conditions (12:12-h light:dark cycle, room temperature of 22 ± 2 °C) and German law. All experimental procedures including blood sample collection were approved by the local committee for ethics and animal welfare and the responsible authority of the district government of Upper Bavaria. As part of the GMC primary phenotyping screens, blood samples were taken in the morning from isoflurane-anesthetized mice by retrobulbar puncture without prior food fasting and collected in Li-heparin-coated sample tubes (Kabe Labortechnik, Numbrecht, Germany).[33] Sample collection was performed as either a survival sample collection with subsequent fluid replacement in the standard primary GMC phenotyping screen[15,16] or as a final bleed without revival of the mice within the International Mouse Phenotyping Consortium phenotyping pipeline. In the first case, the sample volume collected was 200 to 400 µL, depending on body mass, whereas as much as 700 µL of blood were collected during final bleeding. Samples were stored at room temperature for 1 to 2 h before being separated by centrifugation. Heparinized plasma samples were processed immediately after separation or stored at -20° or -80 °C for not more than 1 mo before analysis. The numbers

Table 2. Comparison of female and male mice

	Female	Male
Sodium, mmol/L	146 (143–148)	148 (146–151)
Potassium, mmol/L	3.8 (3.5–4.1)	4.2 (3.8–4.4)
Calcium, mmol/L	2.30 (2.22–2.37)	2.34 (2.26–2.40)
Chloride, mmol/L	109 (107–111)	109 (107–111)
Phosphate, mmol/L	1.4 (1.2–1.8)	1.4 (1.2–1.8)
Total protein, g/L	49.8 (47.5 – 52.0)	50.0 (48.0–53.3)
Urea, mmol/L	10.62 (9.27–12.09)	10.90 (9.86–12.05)
Cholesterol, mmol/L	1.96 (1.77–2.20)	2.59 (2.29–2.90)
Triglyceride, mmol/L	1.09 (0.85–1.39)	1.79 (1.37–2.23)
ALT, U/L	26 (22–32)	30 (24–40)
AST, U/L	50 (44–60)	48 (40–60)
ALP, U/L	135 (122–148)	90 (80–100)
Albumin, g/L	28.0 (26.0–29.0)	26.0 (25.2–28.0)
α-amylase, U/L	558.3 (512.6– 618.9)	669.5 (607.7– 744.2)

Data are given as median (interquartile range). Values differed significantly ($P < 0.001$) between male and female mice for all analytes except phosphate (P = 0.488).

of fresh and frozen samples analyzed per strain or substrain are given in Table 1. Plasma samples were analyzed without dilution (samples collected within the International Mouse Phenotyping Consortium project and representing a majority of C57BL/6NTac mice) or diluted 1:2 with deionized water (samples representing a majority of the samples of the other strains or substrains) by using a clinical chemistry analyzer (model AU400, Olympus, Hamburg, Germany, or model AU480, Beckman-Coulter, Krefeld, Germany). Reagent kits developed for human samples (Olympus or Beckman–Coulter) but adapted for mouse samples were used according to the manufacturer`s instructions. Daily quality controls were performed before sample analysis as recommended.[34] The set of analytes measured routinely changed periodically and was adapted to specific requirements in some projects. We selected for our analysis the analytes with the highest number of measured values available. Glucose levels, although measured in most of the samples, were not included, given that the fed state at sample collection, sample collection in the absence of glycolysis inhibitor, and variable duration of sample storage before plasma separation led to high variability among measured values.

Missing data. Within the 2947 mice in the study population, no data were available for 10 mice that had died before blood collection and one additional mouse that was not bled for reasons of animal wellbeing. For another 4 mice, sample volume or quality was insufficient to perform the analyses. For 16 of the 161 cohorts of mice, only a partial set of analytes was measured due to project-specific adaptations of the standard set of analytes or failure to correctly perform measurements of single analytes due to technical reasons. In addition, values for 1, 2, or 3 analytes were missing in 13 individual mice due to insufficient sample volume or quality.

Statistical analysis. Statistical analysis was performed by using GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA), SPSS Statistics (version 17.0, SPSS, Chicago, IL), and R (version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria). Descriptive and explorative data analyses were conducted for all study variables. Data are presented as boxplots showing median values with interquartile ranges and 90% data ranges. Continuous data meeting the assumption of normal distribution were compared by using the Student *t* test for unpaired samples (sex effects) and across multiple groups by pairwise *t* test with Bonferroni correction (strain or substrain effects). Otherwise the nonparametric Wilcoxon rank-sum test with Bonferroni correction was used. For correlation analyses (age effects), the Pearson correlation coefficient was used for normally distributed data (potassium, chloride, calcium, urea, total protein, and albumin), and the Spearman correlation coefficient was used otherwise (sodium, inorganic phosphate, cholesterol, triglyceride, ALT, AST, ALP, α-amylase). Because large numbers of animals were used, a *P* value less than 0.01 was considered as significant for all tests.

Table 3. Correlation of clinical chemistry markers with age in male and female mice

	Female		Male	
	r	*P*	r	*P*
Sodium	0.17	<0.001	0.11	<0.001
Potassium	0.18	<0.001	0.21	<0.001
Calcium	0.13	<0.001	0.05	0.068
Chloride	−0.03	0.190	0.15	<0.001
Phosphate	0.11	<0.001	0.06	0.015
Total protein	0.29	<0.001	0.27	<0.001
Urea	−0.11	<0.001	0.00	0.930
Cholesterol	0.27	<0.001	0.16	<0.001
Triglyceride	0.11	<0.001	0.23	<0.001
ALT	0.01	0.772	0.03	0.236
AST	0.03	0.332	0.03	0.311
ALP	−0.12	<0.001	−0.13	<0.001
Albumin	0.16	<0.001	0.21	<0.001
α-amylase	0.00	0.937	0.03	0.240

To identify the relationship between sex and strain or substrain, phenotypic data of 14 clinical chemistry variables were used in a hierarchical agglomerative cluster analysis. For this, the mean value of each analyte was calculated for each strain or substrain–sex combination. Because some of the analytes have considerably different ranges between sexes or strains, these values were finally transformed to the Z-score, representing the number of standard deviations a value differs from an overall mean. Here, the Z-score for analyte X of a male or female strain or substrain group Y was calculated as follows:

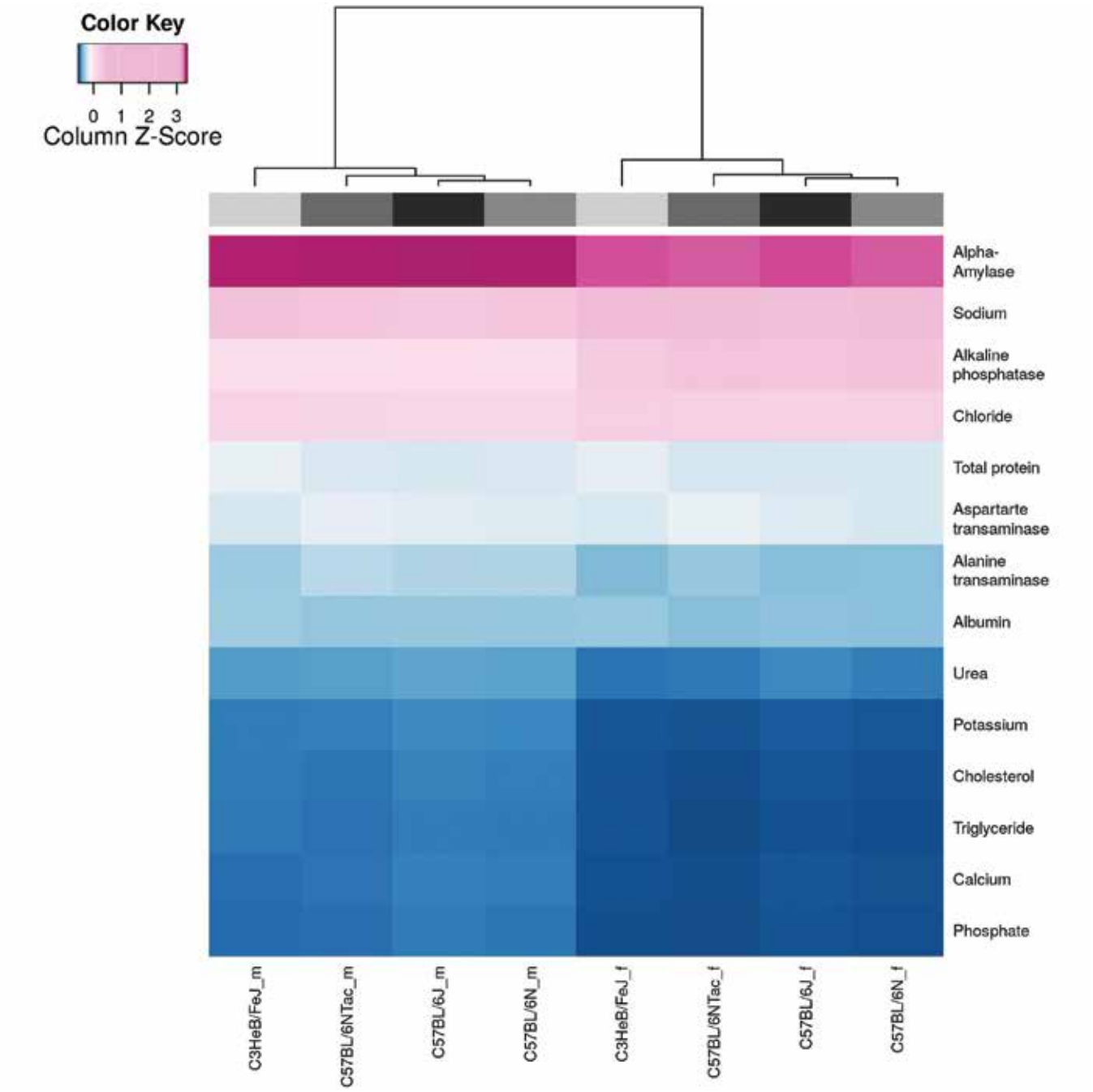

Figure 1. Dendrogram and heatmap of unsupervised hierarchical agglomerative cluster analysis according to the Z-score–transformed calculated means of the clinical chemistry markers. The Z-score transformation was used to put all analytes on a same scale, thus ensuring that analytes with increased variability did not dominate the clustering. The gray colors between dendrogram and heatmap represent the 4 strains and substrains analyzed and were chosen according to the fur color of the strains. A horizontal line in the dendrogram represents a merge of clusters, whereby the *y*-coordinate of the horizontal line represents the similarity of the 2 clusters being merged. The dendrogram can be cut where the gap between 2 clusters is largest. In our case, this process results in 2 large clusters—female and male mice—thus indicating that sex-specific differences are more prominent than are strain effects. Additional separation occurs between strains with the C3H and C57BL/6 backgrounds.

$$Z = (\text{mean of X in group Y} - \text{mean of all analyte means in group Y}) \div (\text{SD of all analyte means in group Y})$$

Because of this transformation over all analytes, each strain or substrain has a mean of 0 and a SD of 1. This process sets the values for the different analytes on similar scales and ensures thereby that analytes with greater variability do not dominate the clustering. Starting from their own single clusters, the Z-score transformed mean values were then merged successively. To decide which clusters should be merged, the distance between the analytes' transformed mean values was calculated through the Euclidean distance at each step. The linkage criterion finally specified which clusters should be combined based on their calculated distance—the ward criterion as used in this study here aims to join the clusters

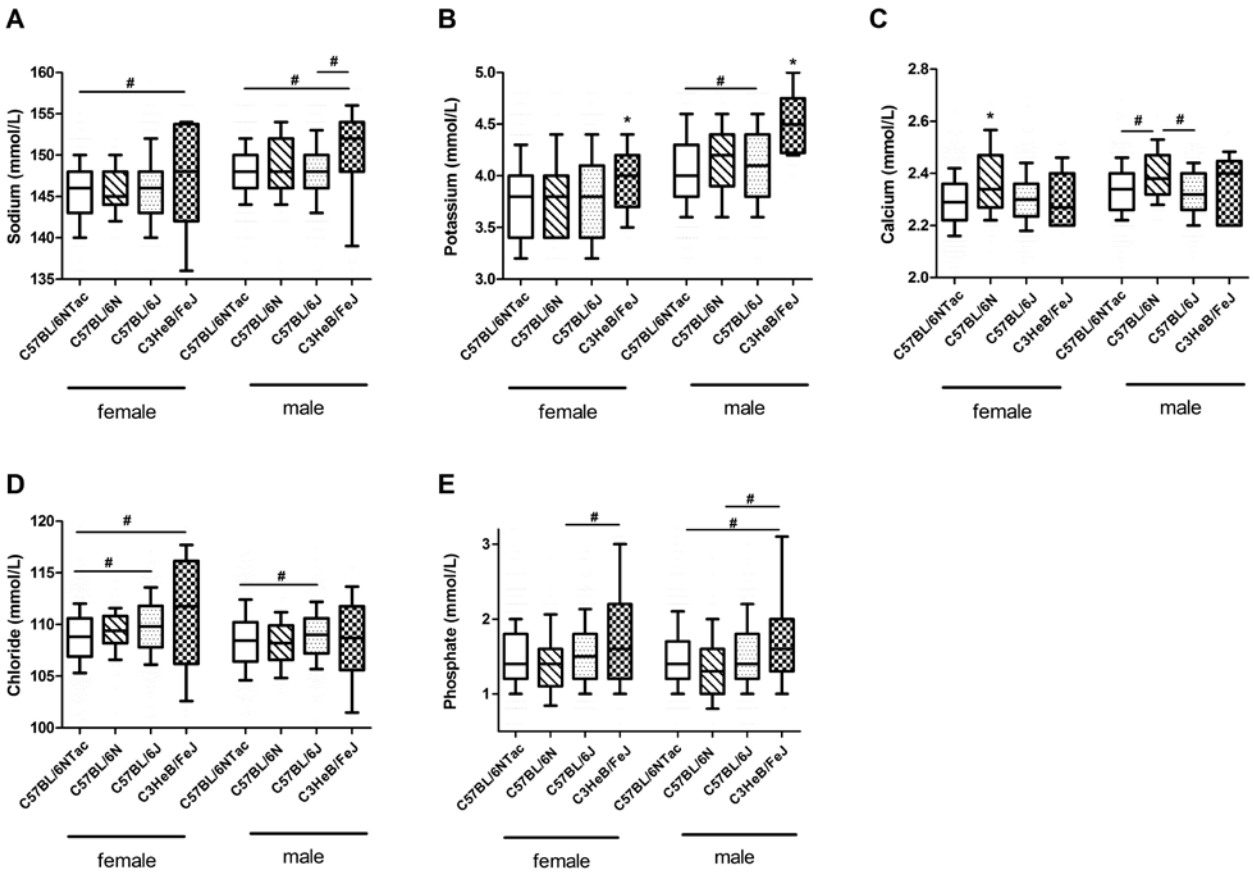

Figure 2. Electrolyte concentrations of 90- to 135-d-old mice according to their sex and strain or substrain. (A) Sodium. (B) Potassium. (C) Calcium. (D) Chloride. (E) Phosphate. Data are depicted as boxplots that indicate the median, 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers). Asterisks (*) indicate values that differ significantly ($P < 0.05$) from those of all other mice of the same sex but different strain or substrain; hashmarks (#) indicate significant differences between the 2 indicated strains or substrains.

which have the smallest within cluster variance. The results of hierarchical agglomerative clustering are visualized as a dendrogram, which allows reconstruction of the process of merging that lead to the presented clustering. An important advantage of visualizing the analytes by means of a heatmap and a dendrogram is that patterns in the data can be identified what cannot be obtained when looking individually at each single analyte of the examined strain or substrain.[1]

Sex-specific reference values for each strain or substrain were calculated and covered 95% of all values of healthy wild-type mice between 90 and 135 d of age.

Literature review. To obtain an overview of published articles that present values of clinical chemistry blood analytes in wild-type mice of different strains, we searched the NCBI PubMed literature database by using the following terms in various combinations: mouse, clinical chemistry, blood, biochemistry, reference values, and normal values.

Results

In total, 2932 mice that met the selection criteria concerning genetic background and availability of data from the clinical chemistry sets of electrolytes (sodium, potassium, calcium, chloride, inorganic phosphate), lipid profile (cholesterol, triglyceride), enzyme activities (alanine transaminase, aspartate transaminase, alkaline phosphatase, α-amylase) as well as urea, albumin, and total protein levels were identified from more than 12,000 mice and are listed according to sex and strain or substrain in Table 1.

Sex-associated effects. To analyze sex-specific effects, data from male and female mice of all strains or substrains were compared. Most analytes showed significant sex-associated differences (Table 2). Overall, female mice presented significantly ($P < 0.001$) higher values of chloride, AST, ALP, and albumin, whereas male mice demonstrated higher ($P < 0.001$) levels for sodium, potassium, calcium, total protein, urea, cholesterol, triglyceride, ALT, and α-amylase. No significant differences between male and female mice were found for inorganic phosphate ($P = 0.488$). In light of these sex-related effects, additional analyses were performed separately for male and female mice.

Age-dependent effects. Correlation analysis demonstrated significant correlations between age and most of the analyzed clinical chemistry analytes (Table 3). Specifically, sodium, potassium, total protein, cholesterol, triglyceride, ALP, and albumin correlated ($P < 0.001$) with age in female and male mice. In addition, significant ($P < 0.001$) correlations with age were found for calcium, urea, and inorganic phosphate in female mice and for chloride in male mice. Although statistically significant, all of these correlations presented with a low coefficient of determination ($R^2 \leq 0.084$), so that their biologic relevance is unclear. Age coverage varied between strains and substrains such that, to provide a homogenous distribution overall, only mice with an age of 90 to 135 d were included in further analyses. This range covers 91% of all identified mice. Within this age range, the described sex-associated effects remained stable.

Strain- and substrain-associated effects. We performed a cluster analysis of female and male mice to investigate the phenotypic relation between the strains and substrains of 90- to 135-d-old

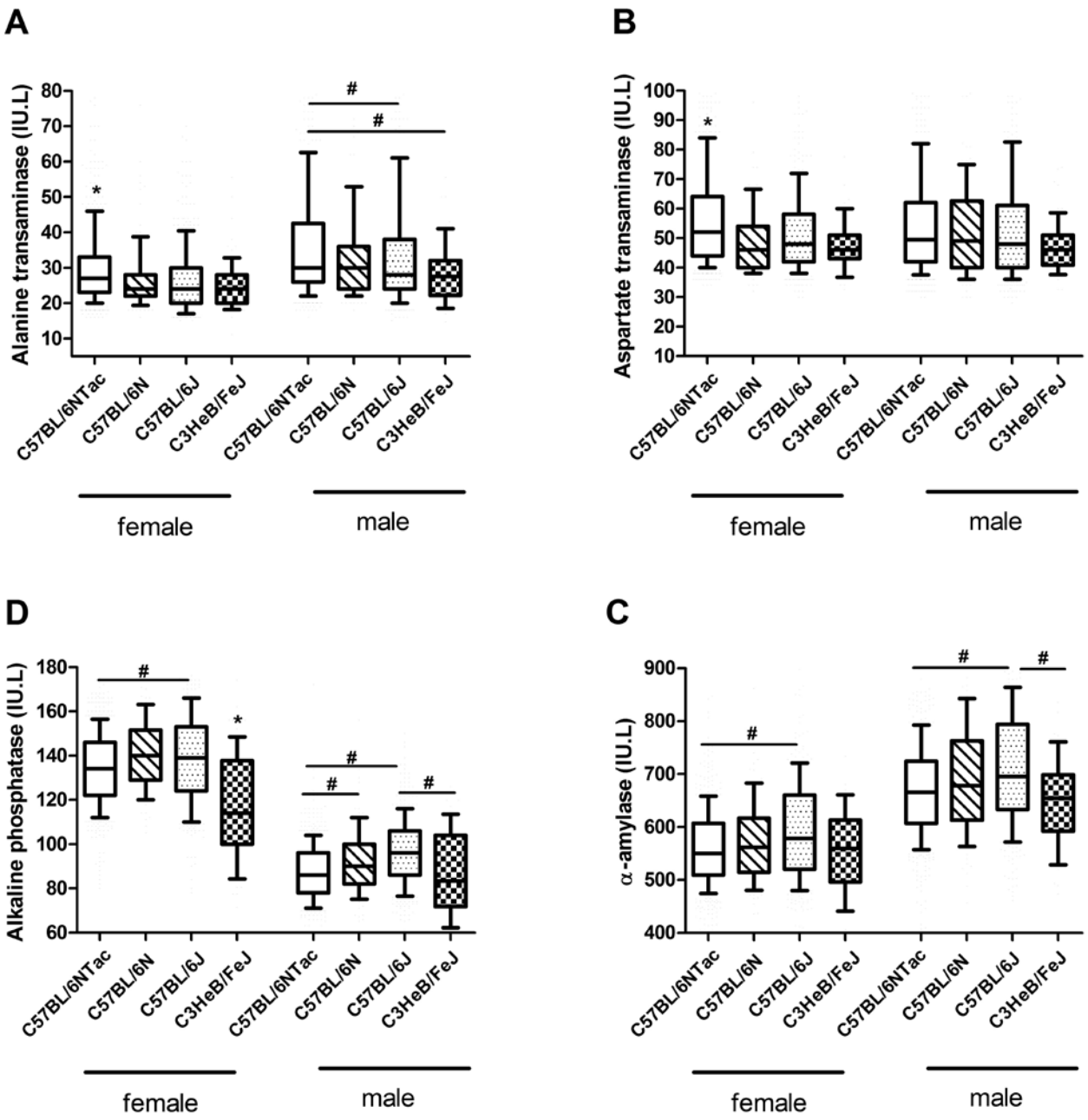

Figure 3. Plasma enzyme activities of 90- to 135-d-old mice according to their sex and strain or substrain. (A) ALT. (B) AST. (C) ALP. (D) α-amylase. Data are depicted as boxplots that indicate the median, 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers). Asterisks (*) indicate values that differ significantly ($P < 0.05$) from those of all other mice of the same sex but different strain or substrain; hashmarks (#) indicate significant differences between the 2 indicated strains or substrains.

mice (Figure 1). Here, the clear separation of male and female groups indicates that sex-specific differences are more prominent than strain effects. For both female and male mice, C57BL/6J and C57BL/6N mice were closely related, with a slightly increased distance for C57BL/6NTac mice. In this analysis, mice belonging to the C3HeB/FeJ strain seem to be more different and were separated from mice belonging to C57BL/6 substrains. In line with this finding, the single comparison of clinical chemistry values for each analyte demonstrated significant differences between the investigated strains and substrains and affected most analytes in both female and male mice—not only between C3HeB/FeJ and C57BL/6 but also between the C57BL/6N, C57BL/6J, and C57BL/6NTac substrains (Figures 2 through 4). Nevertheless, the highest number of significant differences was identified for C3HeB/FeJ mice. For several analytes, including albumin, total protein, potassium, triglyceride, and cholesterol, C3HeB/FeJ mice were clearly distinct from all C57BL/6 substrains, which were more similar to each other in regard to these analytes.

References intervals. The reference intervals determined by standardized clinical chemistry analysis within GMC primary phenotyping for female and male C57BL/6J, C57BL/6N, C57BL/6NTac, and C3HeB/FeJ animals cover 95% of all the values of healthy wild-type mice aged 90 to 135 d and are presented as median and 2.5th and 97.5th percentiles for male mice in Table 4 and for female mice in Table 5.

Literature overview. Several studies published during the past few decades provide clinical chemistry data on various mouse strains. An overview of this literature is given in Table 6.

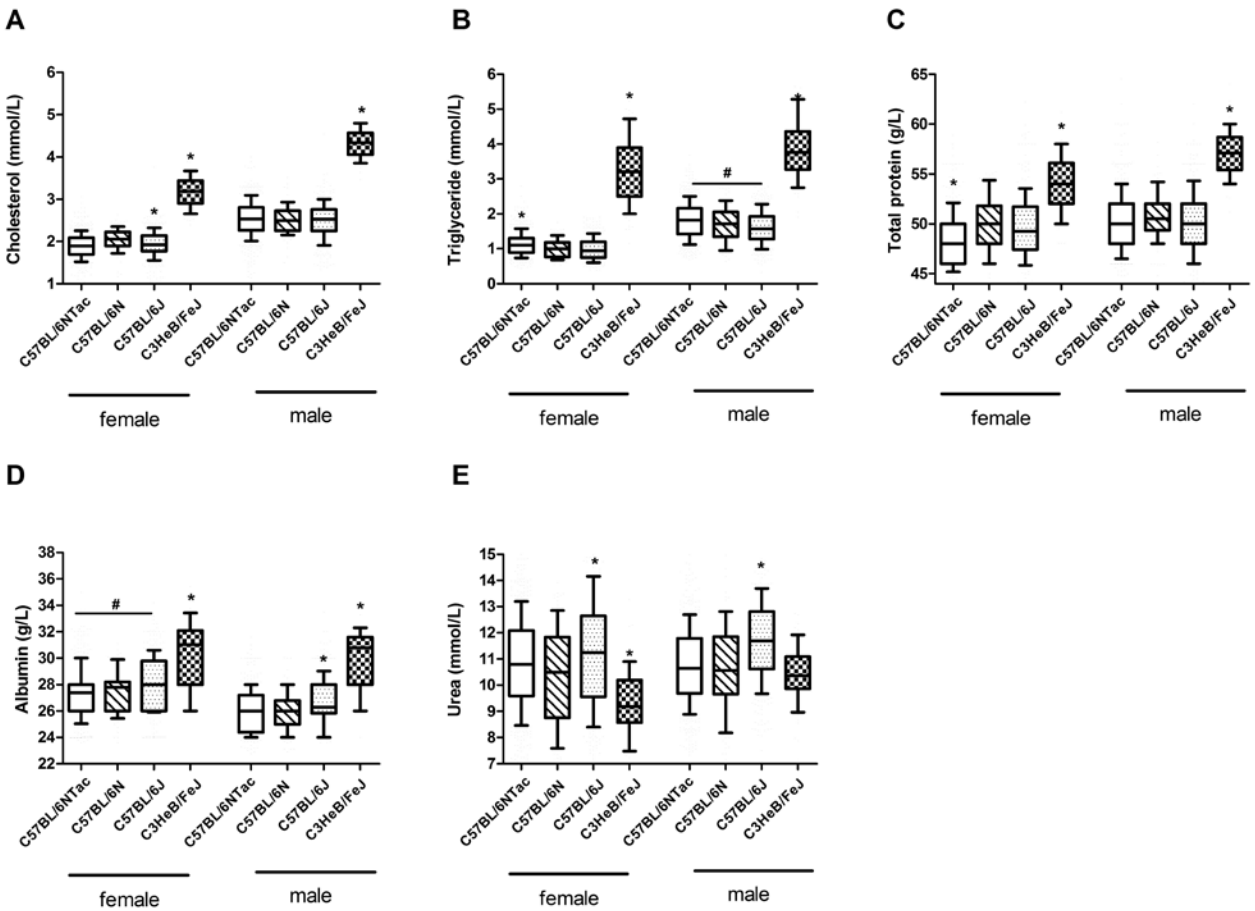

Figure 4. Plasma lipids, proteins, and urea of 90- to 135-d-old mice according to their sex and strain or substrain. (A) Cholesterol. (B) Triglyceride. (C) Total protein. (D) Albumin. (E) Urea. Data are depicted as boxplots that indicate the median, 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers). Asterisks (*) indicate values that differ significantly ($P < 0.05$) from those of all other mice of the same sex but different strain or substrain; hashmarks (#) indicate significant differences between the 2 indicated strains or substrains.

Table 4. Reference intervals for female mice (age, 90 to 135 d)

	C57BL/6N				C57BL/6NTac				C57BL/6J				C3HeB/FeJ			
	n	2.5%	median	97.5%	*n*	2.5%	median	97.5%	*n*	2.5%	median	97.5%	*n*	2.5%	median	97.5%
Sodium, mmol/L	103	140	145	150	705	138	146	152	429	138	146	155	84	134	148	156
Potassium, mmol/L	113	3.2	3.8	4.6	704	3.0	3.8	4.6	420	3.1	3.8	4.6	84	3.5	4.0	4.8
Calcium, mmol/L	113	2.16	2.34	2.61	709	2.09	2.29	2.50	441	2.10	2.30	2.51	84	2.00	2.27	2.51
Chloride, mmol/L	103	105	109	113	697	103	109	114	432	105	110	116	84	102	112	119
Phosphate, mmol/L	113	0.8	1.4	2.3	712	0.8	1.4	2.4	441	0.8	1.5	2.5	82	0.8	1.6	3.1
Total protein, g/L	113	44.9	50.0	55.0	638	43.5	48.0	54.8	434	43.5	49.3	58	74	48.0	54.0	58.7
Urea, mmol/L	113	7.02	10.49	13.46	712	7.28	10.79	14.41	422	7.57	11.25	15.52	84	6.92	9.19	12.29
Cholesterol, mmol/L	113	1.43	2.06	2.48	713	1.25	1.90	2.50	444	1.16	1.93	2.60	84	2.52	3.19	4.03
Triglyceride, mmol/L	113	0.59	0.99	1.55	713	0.59	1.10	1.92	444	0.50	0.94	1.76	84	1.56	3.20	5.21
ALT, U/L	113	18	24	57	713	18	27	67	444	14	24	56	83	18	24	44
AST, U/L	113	34	46	82	713	38	52	114	441	34	48	100	84	34	46	99
ALP, U/L	113	112	140	176	713	102	134	167	441	100	139	184	84	78	114	156
Albumin, g/L	113	24.3	27.8	30.3	712	24.0	27.4	31.6	421	24.0	28.0	32.0	56	26.0	31.1	34.3
α-amylase, U/L	113	462.9	561.9	729.8	657	427.4	550.6	748.9	406	449.0	578.6	865.8	46	430.3	560.0	700.6

Discussion

In our study and using standardized analysis conditions, we determined sex-specific reference intervals for sodium, potassium, calcium, chloride, inorganic phosphate, cholesterol, triglyceride, ALT, AST, ALP, α-amylase, urea, albumin, and total protein levels of healthy C57BL/6J, C57BL/6N, C57BL/6NTac, and C3HeB/FeJ wild-type mice at 90 to 135 d of age. In our experience, it is difficult to identify appropriate published reference values for frequently used clinical chemistry analytes of various inbred mouse strains and to compare these results among each other.[5,8,18,19,38,42,46] The values obtained are strongly dependent on diet, housing, blood sampling procedure, pre-

Table 5. Reference intervals for male mice (age, 90 to 135 d)

	C57BL/6N				C57BL/6NTac				C57BL/6J				C3HeB/FeJ			
	n	2.5%	median	97.5%	*n*	2.5%	median	97.5%	*n*	2.5%	median	97.5%	*n*	2.5%	median	97.5%
Sodium, mmol/L	99	142	148	156	666	140	148	154	416	140	148	156	76	137	152	158
Potassium, mmol/L	109	3.4	4.2	4.8	666	3.4	4.0	4.8	406	3.5	4.1	4.8	76	4.0	4.5	5.2
Calcium, mmol/L	109	2.22	2.38	2.57	666	2.14	2.34	2.52	425	2.16	2.32	2.50	76	2.18	2.40	2.54
Chloride, mmol/L	99	104	108	112	658	102	109	115	425	104	109	114	76	101	109	115
Phosphate, mmol/L	109	0.8	1.3	2.4	673	0.8	1.4	2.5	426	0.8	1.4	2.5	76	1.0	1.6	3.3
Total protein, g/L	109	45.9	50.5	56.5	599	44.8	50.0	58.0	426	44.0	50.0	56.8	59	52.6	57.1	61.6
Urea, mmol/L	109	7.53	10.56	14.24	674	7.82	10.64	13.93	407	8.31	11.70	15.01	76	7.95	10.36	12.39
Cholesterol, mmol/L	109	1.60	2.49	3.27	674	1.65	2.54	3.31	435	1.50	2.53	3.30	76	3.74	4.34	5.08
Triglyceride, mmol/L	109	0.80	1.71	2.73	674	0.85	1.82	2.90	435	0.75	1.57	2.57	76	2.43	3.76	5.73
ALT, U/L	109	20	30	82	673	20	30	96	433	18	28	94	76	18	28	51
AST, U/L	109	31	49	98	674	34	50	138	423	32	48	122	76	36	46	74
ALP, U/L	109	66	90	129	674	60	86	114	425	68	96	132	76	43	83	120
Albumin, g/L	109	23.1	26.0	29.4	673	22.0	26.0	29.8	417	22.0	26.3	30.5	51	26.0	30.8	32.5
α-amylase, U/L	107	517.5	678.2	943.5	616	501.2	666.1	859.6	399	507.3	695.6	962.2	44	489.7	654.4	813.9

analytic handling of samples, equipment and methods used for analyses, and other factors[7,9,11,20,21,32,33,37,44] In particular, the ongoing development and improvement of the analytic methods necessitates regular updates of reference values. For example, our data for C57BL/6J mice were in a range similar published values[38] for mice of approximately the same age, although the mice in the previous study belonged to different strains and were analyzed by using different equipment. In contrast, the comparison also suggests that differences with our results (for example, values for electrolytes) might be related to differences in the methodology or small cohort size (7 to 9 animals per sex) used previously.[38]

To check for similarity between data collected by using the same methods, we compared our results to a subset of data selected from a previous study of our lab and 2 studies from the Institut Clinique de la Souris;[7,8,23] these data were obtained from C57BL/6J and C3HeB/FeJ mice and were investigated by using the same technology as in the current study. Data given in different units were converted to the units used in our study, and 25 °C enzyme activities[23] were converted to 36 °C values. However, the experimental conditions of these earlier studies did not fully match those of our present study, in that the mice investigated previously were younger than those we used for the determination of the reference values and underwent 2-h or overnight food deprivation before sample collection.[7,8,23] Because the earlier studies reported only means and SEM whereas we here present the 2.5th and 97.5th percentiles to define the normal interval, a direct statistical comparison was not possible. However, to estimate whether the data obtained from the previous and current studies fell within a similar interval, we compared the position of the confidence intervals of the means (mean ± 2 × SEM) for each analyte analyzed in both the previous and current study with our reference interval for the respective strain and sex (Figure 5).

Given the differences in experimental procedures and statistical evaluation of data, some deviations are expected, but the mean and median in a data set are expected to be similar between the previous and current studies, provided that the data are normally distributed. However, in a right-skewed distribution, where most of the values are rather low and therefore concentrated on the left and where only few animals with elevated values form a long tail on the right side, the mean would be higher than the median value—most of the liver enzyme activities in the previous studies[7,8,23] displayed this pattern. We also anticipated higher ALP activities in younger mice, given that this analyte usually shows decreasing activity with increasing age, at least during the first 6 mo of life.[8] In addition, the α-amylase activities in the previous studies[7,8,23] are expected to be similar or higher than those in our study, given the increase in α-amylase activities with increasing fasting duration in C57BL/6J mice.[7] Because long-term fasting decreases plasma glucose levels C57BL/6J mice,[7] the glucose levels of the overnight-fasted animals are expected to be decreased relative to our reference levels.

Of the 100 single comparisons, including comparisons of our results with data of as many as 14 parameters from C57BL/6J and C3HeB/FeJ male and female mice from 2 studies each, the confidence interval of the mean from the previous studies[7,8,23] fell within our reference intervals in 62 cases, but in only 7 comparisons was our median value within the confidence interval of the mean. In 19 comparisons, the confidence intervals of the mean were situated at the lower or upper limit of our reference intervals, and in 19 cases the previous mean values were below or above our reference intervals. In many cases, observed deviations were in line with our expectations, such as mean ALT, AST, and ALP activities above our median value. Deviations observed for urea, cholesterol, and triglyceride values seemed to reflect a sex- and strain-specific response to long-term food deprivation. Although these values were similar or increased in C57BL/6J mice, mean values in both comparisons of overnight fasted C3HeB/FeJ mice with our reference interval were near or below the lower limit of our reference interval. In addition to these deviations, the phosphate levels of 2-h fasted C57BL/6J mice and the mean potassium values of male C57BL/6J and male and female C3HeB/FeJ mice analyzed at the Institut Clinique de la Souris were higher than the reference values we determined. The detailed comparison of results from selected previous studies to our reference intervals demonstrates that data collected from the same inbred strains of mice using the same analytical system are situated within similar ranges.

Table 6. Overview of literature presenting clinical chemistry data from several mouse strains

	Mice							
Reference	Strain	Sex	Age	No. per group	Experimental conditions	Sample	Analytical device	Data
7	C57BL/6J	Male and female	9–12 wk	25–100	Comparison of conditions (housing, fasting duration, diet, diurnal rhythm, anesthesia), retrobulbar collection	Heparin-treated plasma, fresh	AU400 (Olympus)	Mean ± SEM
8	C57BL/6J, 129SvPas, BALB/cByJ, C3HeB/FeJ	Male and female	3, 6, and 12 mo	40	Overnight fasting values, isoflurane anesthesia, retrobulbar collection	Heparin-treated plasma, fresh	AU400 (Olympus)	Mean ± SEM
18	B6D2F1	Male	~45 d	304	Single housing, treatments before blood collection, chloroform anesthesia, thoracotomy, cardiocentesis	K_2-EDTA plasma (fresh or frozen)	Basic microanalyzer (Ortho Instruments)	Percentiles, mean ± 1 SD
19	C3H, BALB/c	Male and female	56–78 d	18–26	Samples collected from tail vein	Heparin-treated plasma, fresh	AU400 (Olympus)	Mean ± SEM
23	C3HeB/FeJ, C57BL/6JIco, BALB/c, C3BlF1, B6C3F1, C3CF1, CC3F1	Male and female	12 wk	37–132	Overnight fast, ether anesthesia, retrobulbar collection	Heparin-treated plasma, frozen	Hitachi 717 (Roche) and AU400 (Olympus)	Mean ± SEM
27	C57BL/6J, 129SV/EV, C3H/HeJ	Male and female	2, 4, 6, 8, 10, and 12 mo	15	Isoflurane anesthesia, retrobulbar collection, analysis of pooled samples (3 mice)	Serum, frozen	Vitros 250 (Ortho Diagnostics)	Median and percentiles
36	FVB/NTac	Male and female	7–9 wk	52–54	Microisolation housing, CO_2 anesthesia, cardiocentesis	Serum, frozen	Vitros DTII (Ortho Diagnostics)	Percentiles, mean ± 1 SD
38	FVB/NCrlBR, C57BL/6J-Tyrc2J/+, Swiss Webster (outbred)	Male and female	14–18 wk	16–18	Housing with cage enrichment, 4-h fasting, CO_2 anesthesia, cardiocentesis	Serum, fresh	Hitachi 704 (Boehringer Mannheim)	Minimum, maximum, mean ± 1 SD
42	C3H/HeH, BALB/cAnNCr, C57BL/6J	Male and female	24–30 wk	10–20	Single housing in metabolic cages for 7 d; sample collected from internal jugular vein after euthanasia	Heparin-treated plasma, frozen	AU400 (Olympus)	Mean ± 1 SD

However, this analysis also shows that significant variability can be expected depending on the actual experimental conditions, emphasizing that reference ranges cannot replace the inclusion of suitable controls in each experiment.

Many vendors themselves provide reference intervals or at least mean values for laboratory parameters from different strains or substrains they offer. The precise age and numbers of animals tested, methods used, and statistical distribution of analytes, however, are often insufficiently described or not provided. For example, the mean, SD, and SEM values given on one vendor's webpage[45] are based on results from only 3 to 10 animals per sex per group. In addition, the presented mean liver enzyme activities of C57BL/6NTac mice are much higher than the values we measured here and show greater variation. Charles River Laboratories[24] provide data that reflect larger group sizes ($n \geq 85$), but only for mice that are a maximum of 70 d of age, which is quite young for mice used in animal research. Furthermore, these data were collected by using samples obtained by using cardiocentesis after CO_2 euthanasia, an experimental condition rarely applied in actual research studies. The data presentation by The Jackson Laboratory within the mouse phenome database is very useful and includes data from a broad variety of mouse strains collected in different studies with distinct experimental conditions.[25]

Our study has limitations. First, some of the included mice were obtained from knockout or mutant line studies and rep-

Figure 5. Comparison of confidence intervals of means derived from selected previous studies with the reference intervals determined in the current study. Gray fields indicate missing data or data that cannot be compared (for example, use of different test kits). White fields indicate that the confidence interval of mean of the previous study is situated within our reference interval and includes our median value. Light shades show that the previously published confidence interval lies within our reference interval but is above (pink) or below (blue) our median value. Intermediate shades indicate that the earlier confidence interval partly overlaps our reference interval and includes the upper (pink) or lower (blue) border of our reference interval. Dark shades mark previous confidence intervals that fall entirely above (pink) or below (blue) the reference interval reported in the current study.

resent littermates that were used as wild-type controls and lacking the respective mutation. We included only mice with at least 7 backcrossed generations to wild-type animals of a defined inbred genetic background. However, residual DNA from the other strain might affect some analytes and contribute to increased variation compared with that from purely inbred mice. Although we analyzed large numbers of mice in groups containing as many as 714 animals, the total number of mice within some subgroups might be insufficient; for example, the smallest group—male C3HeB/FeJ mice—contains 76 animals. We decided not to increase this number by increasing the age to maintain similar age ranges for each of the strains. Finally, even with the high degree of standardization applied at our institute, relevant unidentified confounders might exist.

Nevertheless, reference values for analytes of clinical chemistry are necessary to provide initial guidance for researchers. We note that C3HeB/FeJ mice present larger strain-specific phenotypic differences in clinical chemistry analytes than do the closer related C57BL/6J, C57BL/6N, and C57BL/6NTac mice. For example, blood lipid concentrations (cholesterol and triglycerides) as well as protein levels (total protein and albumin) were clearly higher in C3H mice compared with C57BL/6 substrains. These findings represent typical strain-specific differences caused by the distinct genetic background, and similar findings have been obtained in previous studies.[8,23] However, even between C57BL/6N substrains, significant differences were revealed. In general, some markers, such as calcium, potassium, total protein, and inorganic phosphate, seem to be tightly controlled, given the low coefficients of variance for these parameters in previous studies[23] and the low variability in these parameters in our current study. However, other analytes, such as liver enzyme activities, are highly variable, in line with previous results and of clinical relevance.[23,38] For markers that present increased variability, results outside the reference interval may not necessarily indicate a clinically relevant, important change. Nevertheless, the identification of a cohort of mice in which a large proportion yields values beyond the reference range should prompt further investigations, given the likelihood of methodological errors or a relevant abnormal phenotype. Less pronounced changes are perhaps more often related to secondary effects or influencing factors in the mice (for example, infection, variation in food or water uptake) or reflect variability in the experimental procedures (for example, stress response due to mouse handling, preanalytic handling of samples).

In conclusion, this study demonstrates that the definition of 'normal' is age-, sex-, and strain- or substrain-dependent. We here provide reference intervals for several important clinical chemistry analytes of C57BL/6J, C57BL/6N, C57BL/6NTac and C3HeB/FeJ mice and an overview of the literature where additional data can be found. These data analyses should be extended to other strains and markers and should be updated on a regular basis.

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