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Original Research Article

Early View

Body Length Influences Murine Oesophageal Length

Key words

mice;
CD-1;
oesophagus;
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model;
outbred

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Abstract: Mouse models are frequently used in research of oesophageal carcinoma, one of the most prevalent and deadly cancers of the world. Surgical mouse models have been described in a variety of strains and stocks, but the distinct anatomy has not been evaluated before. This is crucial for the investigation of malignant fistulas to the airways, which primarily occur in the mid-oesophagus and within 20 millimetres to the tracheal bifurcation and severely limit survival. We therefore investigated oesophageal and tracheal lengths and their relationship to age, bodyweight, and body length in Crl:CD1(ICR) mice. Using 177 mice, based on an a-priori power-analysis, we found a mean oesophageal length of 38.9 millimetres (standard deviation 4.8) and a mean tracheal length of 16.1 millimetres (standard deviation 1.9). This resulted in a position of the carina relative to the oesophagus at 42% of the oesophageal length. Only body length had a statistically significant association to oesophageal length (t=9.56, P<0.001) in a multivariable regression with an adjusted R^2 of 0.53, but not age (t=1.53, P=0.13) or bodyweight (t=1.05, P=0.3). All three parameters age (t=2.87, P=0.005), bodyweight (t=3.02, P=0.003), and body length (t=2.7, P=0.008) were associated to tracheal length, but with a relevantly lower adjusted R^2 of 0.4. Unlike in rats, where it is in the proximal oesophagus, the tracheal bifurcation is in the mid-oesophagus in mice. The relationship of oesophageal and tracheal length to body length might improve standardisation in surgical mouse models and thereby increase their reproducibility.

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Introduction

Oesophageal cancer is among the most prevalent and deadly cancers worldwide(1) and particularly affects the global south.(2) Preclinical models provide relevant insights because they offer the opportunity for both standardisation and manipulation as necessary for the research question.(3) For research in oesophageal cancer, it has been suggested to use a multi-step approach in animals starting in rats, (4) whose results should then be confirmed and mechanistically elucidated in mice before transferral to large animal models.(5) Mouse models whose oesophageal carcinoma is surgically induced have been described without(6–10) and with(11–14) genetic modifications. While rat models are generally generated in outbred Sprague-Dawley and Wistar stocks, (15) such preference is not established in mice: Some use inbred strains such as A/J, (11) 129/Sv, (10) BALB/c, (6) C57Bl/6, (7, 8) whereas others prefer hybrids(9) or Swiss Webster outbred stock.(13, 14).

We aimed to investigate potential predictors of oesophageal length in mice. This might contribute to model refinement based on better standardisation of surgical mouse models of oesophageal cancer beyond the typically used age(10–13), weight(8) or a combination of them.(6, 9) In addition, we aimed to characterise the relative position of the tracheal bifurcation. Its relevance originates from the rare but grave malignant fistula between oesophagus and trachea, which results in a substantially increased mortality.(16)

Materials and methods

Mice of the CD-1 outbred stock, code Crl:CD1(ICR), were supplied in-house by the *Haus für Experimentelle Therapie* at our institution after they had been bought from a vendor before (Charles River, Sulzfeld, Germany). As there is, contrary to the

situation in rats, in which Sprague Dawley and Wistar stocks dominate, (15) there is no consensus whether to use inbred, (6–8, 10, 11) outbred(13, 14) or hybrid(9) mice for oesophageal carcinoma research in mice, we opted for the pragmatic approach to use the outbred stock available in our facility. Our husbandry practices followed standard guideline recommendations and included regular checks by veterinarians and keepers. The microbiologic status of our mice was specific-pathogen free. Temperatures in the facility were 22 °C constantly with a relative humidity between 45 % and 65 %. The air in the room was exchanged 15 times per hour. Artificial lightning was switched on between 7 and 19 o'clock resulting in a 12-hour dark-light cycle. Cages were individually ventilated and mice were housed in groups of up to five per cage. Beddings were of European aspen with chip sizes from two to three millimetres (Abedd Midi Chips, Abedd, Kalnciems, Latvia). Provided enrichments were nestlets made from dust-free pulped cotton fibre (Ancare, Bellmore, United States). Mice also had access to gnawing sticks (Bricks M, Tapvei, Paekna, Estonia). Cages were exchanged once weekly under aseptic conditions. The facility provided autoclaved tap water *ad libitum* and pelleted regular chow (Ssniff V1534-300, Ssniff Spezialdiäten, Soest, Germany) with 67 % carbohydrates, 24 % protein, and 9 % fat.

We sacrificed the mice in type II long narcotic chambers (Tecniplast, Hohenpeißenberg, Germany) by gradually increasing carbon dioxide concentrations and ensured death by additional cervical dislocation. The necropsy followed standard protocols.(17) Body length was measured from the vertex(18) to the base of the tail with the mouse in prone position (Figure 1A). We did not stretch the mouse in any way before measurements. We measured oesophageal length in situ following from distal to the vestibulum oesophagi as the proximal starting point to the gastro-oesophageal junction as the distal end, as demonstrated by *Ni Bhraonain* and co-workers, (19) except that we used an electronic slide gauge (Kynup, Shenzen, China) for the measurements (Figure 2A) with a resolution of 0.01 millimetres and an accuracy of 0.02 millimetres. Bodyweight was measured with an electric scale (Goldwell 1181, Kao, Darmstadt, Germany) with a resolution of 0.1 gram and an accuracy of 0.01 gram (Figure 1B). We also measured tracheal length in situ from immediately distal to the larynx to the bifurcatio tracheae(18) (Figure 2B).

Based on previously published data from rats, we estimated a sample size of 104 mice to be able to show a deviation of the regression coefficient R^2 from zero based on the R^2 of 0.169 from the literature(15) with an α=0.05 and β=0.9. Due to the different species, we also accounted for additional uncertainty by increasing the calculated number by 70%, resulting in a sample size of 177 mice. We used G*Power (version 3.1.9.2) for the sample size estimation.(20)

Statistical analysis was conducted using GraphPad Prism 8 (Dotmatics, Boston, USA). Descriptive statistics provided were mean with standard deviation and range. Correlation analyses relied on Pearson's *R*. The distribution of the normality of the residuals was checked via Kolmogorov–Smirnov test supported by visual analysis of quantil–quantil plots and homoscedasticity. The prespecified multivariable regression of oesophageal length included age, bodyweight, and body length. This was also conducted for tracheal length. We tested for multicollinearity via a correlation matrix and variance inflation factors.

The use of the mice was exempt from ethical approval. National regulations and the directive 2010/63/EU apply only to living animals, whereas our animals were provided dead, as they had been singled out from conservation colonies. German law for the protection of animals exempts all experiments in which laboratory animals are sacrificed to obtain isolated organs from approval by the competent state authority (exact citation: section seven subsection two sentence three of the German law for the protection of animals (German legal citation: "Paragraph sieben, Absatz zwei, Satz drei des Tierschutzgesetzes")).(21)

For transparency, the raw data of our analyses are freely available from Zenodo (https://doi.org/10.5281/zenodo. 19684433).

Results

We included 177 mice, of which 163 were females and 14 males. Their mean age was 120 days (range 12 – 432 days) with a standard deviation of 71 (Figure 3A), a mean bodyweight of 38.2 grams (range 10.2 – 69.3 grams) with a standard deviation of 7.9 (Figure 3B), and a mean body length of 75.4 millimetres (range 39.9 – 99.9 millimetres) with a standard deviation of 9.1 (Figure 3C).

In these mice, we measured a mean oesophageal length of 38.9 millimetres (range 21.3 – 50.4 millimetres) with a standard deviation of 4.8 (Figure 4A) and a mean tracheal length of 16.1 millimetres (range 10.6 – 21.5 millimetres) with a standard deviation of 1.9 (Figure 4B). Compared to the oesophageal length, the trachea ended at 41.7% of the oesophageal length (95% confidence interval 40.8 – 42.5%) with a standard deviation of 0.06 (Figure 3C).

Before the regression analysis, in order to evaluate potential variance inflation by multicollinearity, we evaluated correlation between independent variables and also their correlation to dependent variables. The correlation between the two dependent variables oesophageal and tracheal length was low with R=0.42 (95% confidence interval: 0.29 – 0.54), whereas there was a moderate correlation between oesophageal length and both age and bodyweight (Figure 5). This was also the case for all independent variables and tracheal length (Figure 5). The sole exception from the low to moderate correlation between independent variables and independent and dependent variables was the strong correlation between body length and oesophageal length with R=0.73 (95% confidence interval 0.65 – 0.79) (Figure 5).

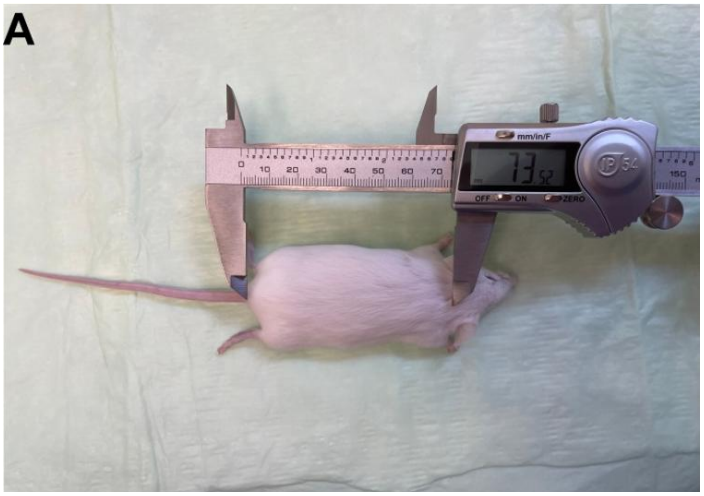

Figure 1: Measurement of body length and body weight. Body length was measured from the vertex to the base of the tail using an electronic slide gauge (A) and body weight was measured using an electronic scale (B). For length measurements, the mouse was placed in prone position and stretch was not applied to the cadaver. For the photographic depiction, the scale is slightly tilted to demonstrate the result of the measurement, whereas it was strictly perpendicular to the surface of the cadaver for the measurement

We then calculated our pre-specified regression model, which had a normal distribution of residuals in the Kolmogorov–Smirnov test and showed no multicollinearity according to the variance inflation factors. Our model was statistically significant for oesophageal length ($F(3, 173)=68.1$, $P<0.001$) and had an adjusted R^2 of 0.533. Only body length was a statistically significant predictor of oesophageal length ($t=9.56$, $P<0.001$), whereas age ($t=1.53$, $P=0.13$) and bodyweight ($t=1.05$, $P=0.3$) were not (Table 1).

Likewise, the model was statistically significant for tracheal length ($F(3, 173)=40.3$, $P<0.001$), but with a lower adjusted R^2 of 0.4. All investigated independent variables were predictors for tracheal length: Age ($t=2.87$, $P=0.005$), bodyweight ($t=3.02$, $P=0.003$), and body length ($t=2.7$, $P=0.008$), but their magnitude was substantially lower than for oesophageal length (Table 2). For example, the influence of body length on oesophageal length is eight times higher in the regression model than for tracheal length.

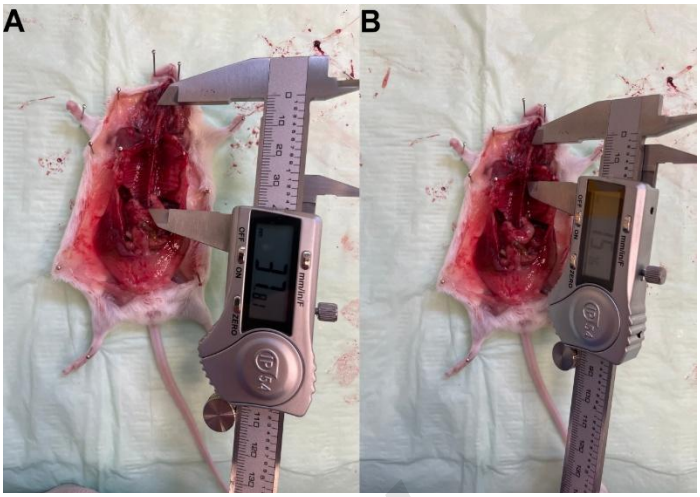

Figure 2: Measurement of oesophageal and tracheal length. The oesophagus was measured from its proximal beginning distal to the vestibulum oesophagi to its distal end at the gastro-oesophageal junction (A). Tracheal length was measured from the proximal beginning immediately distal to the larynx to the distal end at the bifurcatio tracheae (B). As in Figure 1, the scale is slightly tilted to demonstrate the result of the measurement for the photography.

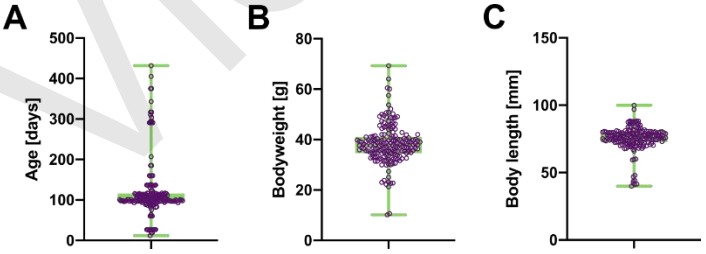

Figure 3: Boxplots of the distribution of measurements of the included 177 mice for age (A), bodyweight (B), and body length (C). The box represents the median and interquartile range, whereas the whiskers extent to the measured maxima and minima. Every circle represents an individual measurement in order to show their distribution. Jitter has been used in the display to avoid overplotting.

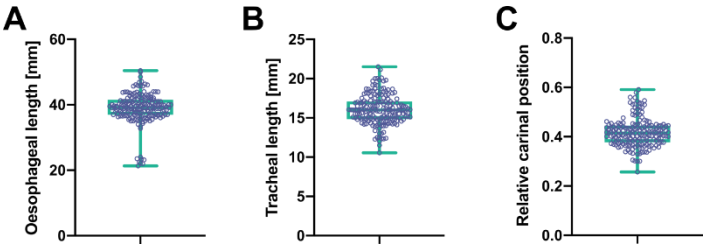

Figure 4: Boxplots of the distribution of measurements of the included 177 mice for oesophageal length (A), tracheal length (B), and the relative position of the tracheal bifurcation to the oesophagus (C). The box represents the median and interquartile range, whereas the whiskers extent to the measured maxima and minima. Every circle represents an individual measurement in order to show their distribution. Jitter has been used in the display to avoid overplotting

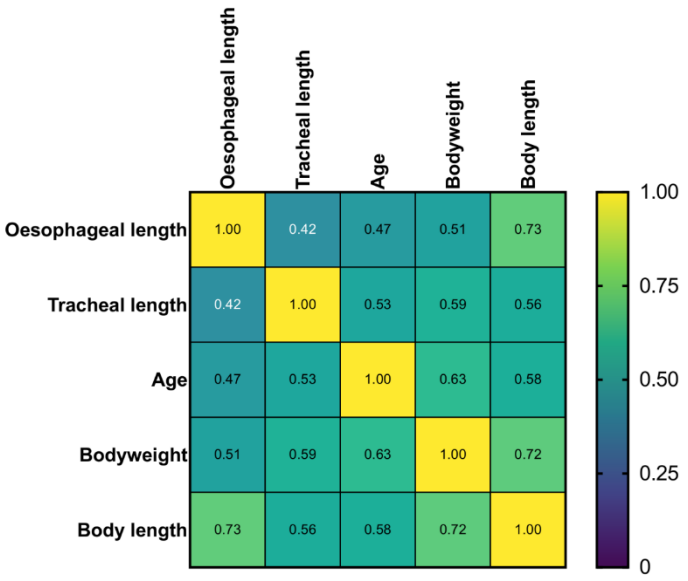

Figure 5: Correlogram of the independent variables oesophageal and tracheal length with the dependent variables age, body-weight, and body length. Correlation coefficients are Pearson's *R* and the number of individual measurements is n=177 Crl:CD1(ICR) mice. The correlogram depicts the respective cor-relation coefficient between two variables. In order to increase clarity, the values of the correlation coefficient have been colour coded

Table 1: Multivariable linear regression of oesophageal length with age, bodyweight, and body length. The model was statistically significant for oesophageal length (F(3, 173)=68.1, P<0.001) and had an adjusted R^2 of 0.53. The residuals were normally distributed (*K*=0.05, *P*>0.1) and variance inflation factors were 1.75, 2.46, and 2.21 respectively. The number of included mice was 177

Variable	Estimate	95% confidence interval	*P*-value
Intercept	11.2	6.8 – 15.6	<0.001
Age	0.007	-0.002 – 0.016	0.13
Bodyweight	-0.05	-0.15 – 0.05	0.3
Body length	0.4	0.3 – 0.5	<0.001

Discussion

We investigated the oesophageal and tracheal lengths in a cohort of Crl:CD1(ICR) mice in order to characterise the anatomical relationship, the relative position of the tracheal bifurcation to the oesophagus, between oesophageal and tracheal lengths. We also aimed to potentially improve standardisation of mice in experiments by investigating whether oesophageal lengths could be predicted by external allometric parameters and therefore tested whether age, bodyweight, and body length were associated to oesophageal length.

Table 2: Multivariable linear regression of tracheal length with age, bodyweight, and body length. The model was statistically significant for oesophageal length (F(3, 173)=40, P<0.001) and had an adjusted R^2 of 0.4. The residuals were normally distributed (K=0.05, P>0.1) and variance inflation factors were 1.75, 2.46, and 2.21 respectively. The number of included mice was 177

Variable	Estimate	95% confidence interval	*P*-value
Intercept	9.1	7.1 – 11.1	<0.001
Age	0.006	0.002 – 0.01	0.005
Bodyweight	0.07	0.02 – 0.1	0.003
Body length	0.05	0.01 – 0.09	0.008

Our study showed that the relative position of the tracheal bifurcation to the oesophagus was at 42% of the oesophageal length. This is in the mid-oesophagus, which is similar to humans, resulting in malignant trachea-oesophageal fistulas arising in mid-oesophageal tumours closely located to the tracheal bifurcation.(22, 23) This is in contrast to the anatomical situation in Sprague Dawley rats (RjHan:SD), in whom the relative position of the tracheal bifurcation is at 31% of the oesophageal length.(24) This study also demonstrated that the relative position of the tracheal bifurcation did not change with increasing age.(24) One may therefore speculate that this could also be the case in mice, which is of relevance as we conducted our investigations in rather old mice, compared to the typical age at surgery for the creation of the model of six to eight weeks.(6–10) As in our study using mice, the extent of variability in rats was even lower with a 95% confidence interval ranging from 29.6% to 31.6% and a standard deviation of 0.027 in 30 rats aged between 57 and 59 days.(24) The lower variability in this very homogenous cohort of rats was expected compared to our cohort, which comprises of a larger age span and thus an a-priori higher variability was expected. Due to the lower variability in the rat cohort, the fundamental difference, the carinal position being not mid-oesophageal as in humans, remains robust.

Moreover, this is also relevant for the successful use of the model, which requires survival of up to 80 weeks in both mice(11, 25) and rats.(26) This finding in rats therefore indicates that the age distribution in our cohort might thus be a lesser problem, but is nonetheless a limitation of our study. The advantage of the mouse over the rat model due to the differing relative position of the tracheal bifurcation arises from the fact that in humans oesophago-mediastinal fistulas arose more often from proximal tumours whereas oesophago-respiratory fistulas primarily arose from mid-oesophageal tumours, but both substantially limit patient survival.(27) Therefore, both models might be used to investigate different types of malignant fistulas, following the suggestion of *Kapoor* et al. to start experimental research in oesophageal carcinoma in rodents.(5)

We also investigated whether any of the allometric parameters age, bodyweight, and body length could aid in standardisation of the used mice. Typically, mice are standardised based on age, bodyweight or a combination thereof. Nevertheless, this results in substantial variation of anatomical parameters, (28) which may result in smaller effects not being detected. In humans, oesophageal length had traditionally been calculated using Strobel's formula with using height alone, (29) but this often resulted in an over-estimation that could be corrected by using body length.(30) We therefore chose these parameters in our investigation, because they are easily measureable in rodents and could therefore potentially be additional tools in standardisation of mice for experiments. Our study demonstrated that this role could only be fulfilled by body length, but not by the commonly used standardisation according to bodyweight or age. The lack of a relevant effect of age and bodyweight on oesophageal length in a regression analysis has also been demonstrated in eight week old Sprague Dawley rats (RjHan:SD) before.(15) This might improve standardisation beyond the commonly used methods and thereby contribute to the uncovering of smaller effect size results without the necessity to increase the number of mice used in experiments and increase reproducibility of results.

A limitation of our study is the age and sex distribution of the included mice. We did not impose sex restrictions following relevant recommendations.(31) This however led to an overrepresentation of females, as males are still more commonly used in experimental research.(32) We intentionally did only include mice in our study that were planned for sacrifice due to non-inclusion in other research projects. We felt it would not be ethically responsible to breed and kill mice solely for our exploratory research project, which could be avoided using said mice. However, this also resulted in a cohort with a variable and, compared to commonly used ages in experimental research, rather old cohort. However, as the mice used in oesophageal cancer research are regularly followed for a year or more, (7, 8, 11, 25) we felt that would be an acceptable limitation at the current stage of investigation. Nonetheless, our study would need a replication in mice at the typical age of surgery. Due to our results with a substantially higher R^2 than the one observed in rats, the number of mice necessary in this future study is substantially lower with 20 mice for 95% power at an α of 0.05. This future study might also offer the opportunity to compare the outbred stock used in our study, based on the assumption that the coefficient of variation should be similar to inbred strains, (33) to typically used inbred strains.

Another limitation of our study originates from the methodologic approach: We used cervical dislocation to ensure deaths after the mice were euthanised via carbon dioxide inhalation, but this may have altered length measurements of the body. Therefore, retrospectively, exsanguination to ensure death would have avoided this potential confounding factor in body length measurement.

Taken together, our study showed that the relative position of the tracheal bifurcation is mid-oesophageal in mice at a level of 42% of the oesophageal length and that oesophageal length was associated to body length only, but not age or bodyweight. This suggests that particularly research addressing malignant fistulas might benefit from standardisation to body length instead of age or body weight.

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Izvleček:

Ključne besede: