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Original scientific article

Tracheal bifurcation located at proximal third of oesophageal length in Sprague Dawley rats of all ages

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Summary

Levrat's rat model is often the first choice for basic studies of oesophageal adenocarcinoma. The position of the tracheal bifurcation represents the preferred location for the high-intrathoracic anastomosis following oesophagectomy for cancer and is thus of importance in basic research of oesophageal adenocarcinoma. In addition, it is also the typical location for trachea-oesophageal fistulae in congenital oesophageal atresia and its rat model. We thus analysed whether the position of the tracheal bifurcation would be affected by a rat's growth throughout life. We analysed absolute and relative carinal position of the tracheal bifurcation and its relationship to oesophageal length in two cohorts of Sprague Dawley rats (RjHan:SD) of both sexes: one consisted of 30 eight-week old rats and the other of 20 rats aged between 15 and 444 days. We analysed their relationship by Pearson's r and univariate linear regression. Bootstrap confidence intervals were calculated for all calculated coefficients. Absolute carinal position correlated with oesophageal length in the eight-week old cohort (r=0.4, 95% CI: 0.08-0.71, p=0.015) and those of different ages (r=0.92, 95% CI: 0.77-0.96, p=0.0066). Absolute carinal position increased with oesophageal length in both cohorts (F(1,28)=5.56; p=0.0256 and F(1,18)=94.93; p<0.0001 respectively). Consequently, relative tracheal bifurcation position was not influenced by oesophageal length in both cohorts (F(1,28)=2.49; p=0.1257 and F(1,18)=1.92; p=0.183). Absolute carinal position increased with oesophageal length, but relative position remained constant at around 30% of proximal oesophageal length throughout life.

Introduction

Experimental studies are often hampered by small sample sizes and low statistical power resulting in over-exaggerated effects and non-reproducibility of their results in humans (Ioannidis 2017). Basic research is however often the first step to advance understanding and therapy of human diseases. Levrat's now iconic rat model of oesophageal adenocarcinoma (Levrat et al. 1962) offers the opportunity to conduct basic research into this carcinoma, whose pathophysiologic mechanisms may then be investigated in mice and thereafter tested in large animal models before transferal to patients (Kapoor et al. 2015). Critically, animal models should be designed to be as close as possible to the specific clinical situation they mimic (Festing and Altman 2002) (e.g., exploratory vs. confirmatory). In this respect, the relative position of the tracheal bifurcation to the oesophagus is of particular relevance in rat models of oesophageal atresia as this level typically determines fistula location (Diez-Pardo et al. 1996). In addition, this level also represents the preferred location for the high intrathoracic anastomosis following oesophagectomy (Yuan et al. 2015) which is used also in rat models (Man et al. 1988). This prompted us to investigate carinal position of the tracheal bifurcation relative to oesophageal length in order to contribute towards the design of reliable and translatable models of oesophageal diseases and surgery.

Materials and Methods

To reduce numbers of experimental animals used in research, data for this study were obtained from two cohorts of rats used in other studies. One study describes a linear relationship between bodyweight and oesophageal length and linear breaking strength in a cohort of 20 rats aged 15 to 444 days with two animals per investigated age (Oetzmann von Sochaczewski et al. 2019a). The other study investigated linear breaking strength of native oesophagi, those with a simple interrupted suture anastomosis, as well as oesophageal suture holding capacity in 30 rats at an age of eight weeks (Tagkalos et al. 2019).

All rats were outbred Sprague Dawley (RjHan:SD) of both sexes obtained from Janvier Labs (Le Genest-Saint-Isle, France). They were housed in our closed-facility under standard husbandry conditions in groups of up to three animals per type-IV cage. The rats were provided with nest material made of European aspen (*Populus tremula*) sized two to three millimetres (Abbedd midi, Vienna, Austria), a tunnel and environmental enrichments in the form of a red polycarbonate house and four tissue papers of two gram each. Rats had access to sterilised standard rat-chow (ssniff Ratte/Maus-Haltung Extrudat, ssnif-Spezialdiäten, Soest, Germany) and water *ad libitum*. A dark-light-cycle of 12 hours starting at 07:00 was employed in a room with 67% relative humidity at 23°C and an air exchange of twelve times per hour. Our experiments complied with the directive 2010/63/EU (European Union 2010) and its subsequent national regulations for the protection of animals. The 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 7 subsection 2 sentence 3 of the German law for the protection of animals [German citation: § 7 II 3 TierSchG]).

Rats were humanely killed by slowly increasing carbon dioxide concentrations and had their oesophagus explanted, in a cluster of viscera with the upper airways, using anatomical landmarks as described elsewhere (Oetzmann von Sochaczewski et al. 2019b). The oesophagus and upper airways were dissected free of surrounding tissue, the carinal position of the tracheal bifurcation was then marked on the oesophagus after which the airways were removed leaving the oesophagus for length measurements using an electronic slide gauge (VWR, Darmstadt, Germany) with a resolution of 0.01mm and an accuracy of 0.03mm as depicted previously (Oetzmann von Sochaczewski et al. 2019c). The carinal position of the tracheal bifurcation was measured from the proximal end of the oesophagus.

We used data obtained from exploratory investigations of five animals per group to conduct a power analysis using G*Power (version 3.1.9.2) (Faul et al. 2007) for a significant deviation of the regression line's slope from zero with a statistical power of 80% and an alpha-level of five percent for the relationship between oesophageal length and relative carinal position in both cohorts. In both cases, a sample size of more than 10,000 oesophagi would be necessary to find a significant deviation from zero, which indicates an extremely small difference without biological relevance. Consequently, using the previously described cohorts is suitable to describe the relationship between oesophageal length and relative carinal position.

Correlation and regression analysis was conducted using R (version 3.5.1) with its stats4-package (R Core Team 2019). Normality of cohorts had been ensured using the Shapiro-Wilk-test (Oetzmann von Sochaczewski et al. 2019a; Tagkalos et al. 2019). To improve precision of our regression analysis, we conducted a bootstrap regression analysis using the simpleboot-package (version 1.1-7) (Peng 2019). In addition, we also bootstrapped a bias-corrected, accelerated (Efron 1987) 95% confidence interval of the mean relative carinal position using the boot-package (version 1.3-22) (Canty and Ripley 2019) and constructed bias-corrected, accelerated bootstrap 95% confidence intervals for correlation coefficients using the wBoot-package (version 1.0.3) (Weiss 2016). All bootstrap-procedures were based on 10,000 repetitions (Baumgart et al. 2020).

Results

Mean relative carinal position of the tracheal bifurcation, as measured from the proximal end of the oesophagus, was at 30.1% of oesophageal length (95% confidence interval: 29.6–31.5%) in rats aged eight weeks (n=30). In this cohort, oesophageal length and absolute carinal position were correlated with r=0.4 (95% confidence interval: 0.08–0.71; p=0.015) and it increased with oesophageal length (Figure 1A). As a result, the relative carinal position did not correlate with oesophageal length (r=-0.29, 95% confidence interval: -0.58–0.17; p=0.203) and did not change with increasing oesophageal length (Figure 1B).

Oesophageal length and absolute carinal position were highly correlated in animals of different ages with r=0.92 (95% confidence interval: 0.77–0.96; p=0.0066) and absolute position also increased with oesophageal length (Figure 2A). Similar to the other cohort, there was no correlation of oesophageal length with relative carinal position (r=-0.31, 95% confidence interval: -0.66–0.32; p=0.279), which also did not change with increasing oesophageal length (Figure 2B).

Discussion

The location of the tracheal bifurcation is of particular importance in oesophageal surgery: in neonates born with oesophageal atresia it often determines the location of trachea-oesophageal fistulas (Spitz, 2007) and in adults the anastomosis following oesophagectomy (Yuan *et al.* 2015). Incidentally, this is also the segment of the oesophagus with the lowest vascular and blood supply (Lister 1964; Oetzmann von Sochaczewski et al.; 2019d; Treutner et al. 1993). Fistula location and anastomotic site also play important roles in rat models of oesophageal diseases and sur-

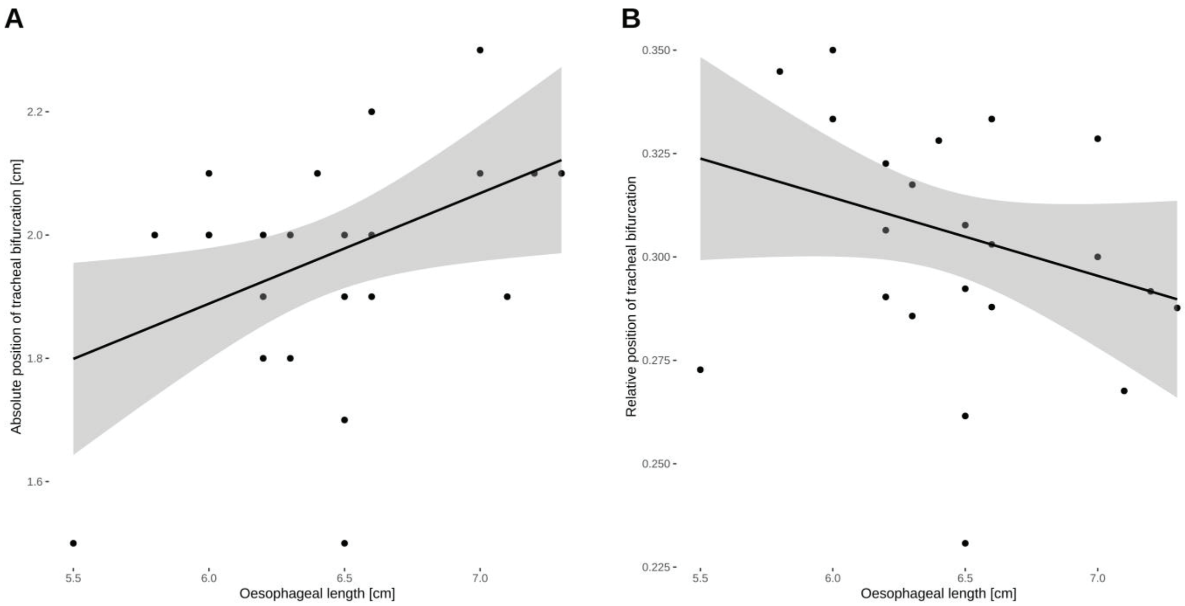

Figure 1. Linear univariate regression of oesophageal length for absolute and relative position of the tracheal bifurcation in 30 rats of eight weeks age.

A. Absolute carinal position of the tracheal bifurcation increases with oesophageal length. Position of the tracheal bifurcation = 0.81 (95% confidence interval: -0.17–1.86) + 0.18 (95% confidence interval: 0.02–0.33) x oesophageal length in centimetres. The model was statistically significant with $F(1,28)=5.56$; p=0.0256 and had an adjusted R^2 of 0.136.

B. Relative carinal position is not influenced by oesophageal length. Relative position of the tracheal bifurcation = 0.43 (95% confidence interval: 0.27–0.6) – 0.019 (95% confidence interval: -0.046–0.005) x oesophageal length in centimetres. The model was statistically insignificant with $F(1,28)=2.49$; p=0.1257 and had an adjusted R^2 of 0.049.

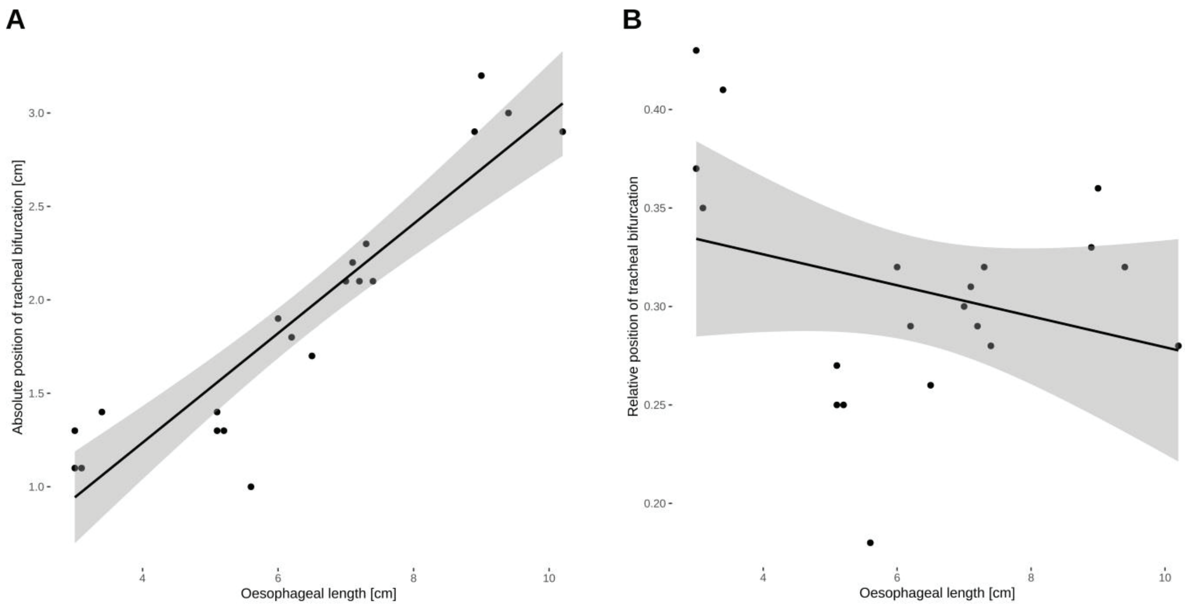

Figure 2. Linear univariate regression of oesophageal length for absolute and relative position of the tracheal bifurcation in 20 rats aged 15 to 444 days.
A. Absolute carinal position of the tracheal bifurcation increases with oesophageal length. Position of the tracheal bifurcation = 0.07 (95% confidence interval: -0.52–0.39) + 0.29 (95% confidence interval: 0.24–0.37) x oesophageal length in centimetres. The model was statistically significant with $F(1,18)=94.93$; $p<0.0001$ and had an adjusted R^2 of 0.832.
B. Relative carinal position is not influenced by oesophageal length. Relative position of the tracheal bifurcation = 0.36 (95% confidence interval: 0.24–0.43) – 0.008 (95% confidence interval: -0.02–0.008) x oesophageal length in centimetres. The model was statistically insignificant with $F(1,18)=1.92$; $p=0.183$ and had an adjusted R^2 of 0.045.

gery (Diez-Pardo et al. 1996; Man et al. 1988). As rats grow throughout their lives (Eisen 1976; Pahl 1969) we investigated whether airways would develop in a corresponding manner to keep a constant positon relative to the oesophagus or have a smaller growth rate resulting in a more cranial location of the tracheal bifurcation. Similarly, we analysed whether the carinal position would be relatively constant at the typical age of eight weeks of surgical models.

While Levrat's initial experiments only lasted a month (Levrat et al. 1962) it is now common to extend experiments to up to 80 weeks (Gronnier et al. 2013). It therefore needs to be ensured that anatomical positions remain comparable throughout study periods as malignant trachea-oesophageal fistulae may develop. Their investigation is of particular clinical interest because a malignant trachea-oesophageal fistula shortens life expectations from months or years to just weeks (Shamji and Inculet 2018). In contrast to post-oesophagectomy trachea-oesophageal fistulae, which are inevitably linked to anastomotic leaks (Lindner et al. 2017), not much is known about their malignant counterparts (Shamji and Inculet 2018). A similar relationship is present in oesophageal atresia: genetics and associated anomalies have been investigated thoroughly (Bogs et al. 2018; Zhang et al. 2017), but not much is known about recurrent trachea-oesophageal fistulae and risk factors for their occurrence (Smithers et al. 2017). This highlights the importance of relative anatomical positions, in particular if these grave complications are investigated in rats as the first choice model of basic research (Kapoor et al. 2015).

Our results demonstrate that the airways grow proportionally to the oesophagus to maintain the relative position of the tracheal bifurcation at the distal end of the proximal third of oesophageal length. A word of caution is however necessary as our finding may not be valid in all rat strains: it has previously been demonstrated that Sprague Dawley rats have higher and organ weights than Fischer 344 rats (Schoeffner et al. 1999). Consequently, the relationship between airways and oesophagus found in Sprague Dawley rats may not be the same in other strains, particularly those with different growth characteristics. In Sprague Dawley rats, proportional growth of oesophagus and airways ensures reliability of the model in terms of anatomical location for a variety of research topics ranging from oesophageal atresia in the neonate to oesophageal malignancy in geriatric patients.

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This study was conducted without funding and we have nothing to disclose.

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