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Extreme selection in humans against homeotic transformations of cervical vertebrae

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EXTREME SELECTION IN HUMANS AGAINST HOMEOTIC

TRANSFORMATIONS OF CERVICAL VERTEBRAE

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ABSTRACT

Why do all mammals, except for sloths and manatees, have exactly seven cervical vertebrae? In other vertebrates and other regions, the vertebral number varies considerably. We have investigated whether natural selection constrains the number of cervical vertebrae in humans. To this end, we have determined the incidence of cervical ribs and other homeotic vertebral changes in radiographs of deceased human fetuses and infants, and analysed several existing datasets on the incidence in infants and adults. Our data show that homeotic transformations that change the number of cervical vertebrae are extremely common in humans, but are strongly selected against: almost all individuals die before reproduction. Selection is most probably indirect, caused by a strong coupling of such changes with major congenital abnormalities. Changes in the number of thoracic vertebrae appear to be subject to weaker selection, in good correspondence with the weaker evolutionary constraint on these numbers. Our analysis highlights the role of prenatal selection in the conservation of our common body plan.

The exceedingly low level of interspecific variation in the number of cervical vertebrae of mammals has puzzled biologists for more than 150 years (E.g. Cuvier 1835; Flower and Lydekker 1891). In birds, reptiles and amphibians, this number varies considerably, and in mammals the number of vertebrae in other vertebral regions is variable as well (Fishel 1906; Galis 1999; Narita and Kuratani 2005). Thus, there appears to be an evolutionary constraint on variations in the mammalian cervical region. Earlier we have suggested that this constraint is the result of selection against deleterious pleiotropic effects associated with variations in the number of cervical vertebrae (Galis 1999). According to this hypothesis selection is, thus, indirect: variations of the number of cervical vertebrae are not disadvantageous as such, but they are associated with other disadvantageous changes. Support for the hypothesis comes from the observation that changes in the number of cervical vertebrae appear to be associated with an increased susceptibility to pediatric cancers, congenital abnormalities and still births in humans and mice (Gladstone and Wakeley 1932; Adson and Coffey 1947; Schumacher et al. 1992; Keeling and Kjaer 1999; Galis and Metz 2003; Merks et al. 2005; Steigenga et al. 2006). In addition, the number of cervical vertebrae is determined during the early organogenesis stage and the conservation of this stage is thought to be the result of selection against deleterious pleiotropic effects of mutations that have an effect on this stage (Sander 1983; Raff 1994; Galis and Metz 2001).

To test this hypothesis, one needs a quantitative study in which the strength of selection against changes of the number of cervical vertebrae is measured and compared with the strength of selection against a less effectively constrained evolutionary change, e.g. changes of the number of thoracic vertebrae. In addition, one needs to establish whether the observed selective disadvantage is indeed the result of deleterious pleiotropic effects that are associated with changes in the number of cervical vertebrae.

To measure the strength of the selection against variations in the number of cervical and thoracic vertebrae we have analysed in humans to which extent variations in the number of cervical and thoracic vertebrae are more prevalent in deceased fetuses and infants than in individuals that survive to a reproductive age. We have screened 598 fetuses and infants that died between 1992 and 1999 in the VU Medical Center in Amsterdam for variations in cervical and thoracic vertebral number and compared the results with existing data on prevalence of vertebral variations in the population at large. Furthermore, to investigate whether selection against vertebral variations is due to pleiotropic effects, we have tested for significant associations between variations in vertebral numbers and congenital abnormalities with deleterious effects.

MATERIAL AND METHODS

Radiographs

Since 1980 all fetuses and infants that are presented for autopsy at the VU Medical Centre in Amsterdam have been standardly radiographed (23mA, 70-90 kV, 4-12 sec, Agfa Gevaert D7DW Structurix films, Figure 1) both ventrally and laterally. We analysed all radiographs made from 1992-1999 (598 cases). In the analysis, we only included fetuses older than 14 weeks, since this is the earliest stage at which ossification centres of cervical ribs can reliably be detected in radiographs (Noback and Robertsen 1951; McNally et al. 1990).). In total, 30 fetuses were excluded from analysis because of insufficient ossification. Fetuses from abortions induced for medical reasons (fatal abnormalities) have been included, except for the calculation of the effect of age at death. Furthermore, infants were analysed that died before the age of one year. At least two people independently analysed each radiograph for variations of vertebral numbers, without prior knowledge of the autopsy reports (however, several congenital anomalies

can be seen in radiographs). Radiographs that were difficult to interpret, or where the interpretation differed between observers, were excluded (44 cases for cervical ribs). Difficulties in interpretation of vertebral variations were due to either insufficient contrast, or because the scapula, maxilla or teeth were obstructing the view of potential cervical ribs. When a cervical rib could only be documented on one side, radiographs were excluded for the scoring of bilateral symmetry (20 cases) and radiographs with an obstructed view of the 19th vertebrae were excluded for the scoring of absent or rudimentary twelfth ribs (4 cases). Absent or rudimentary ribs on the 19th vertebra were scored as absent or rudimentary 12th ribs, except when a cervical rib was also present (since somites from which the vertebrae develop arise and are being patterned in rostro-caudal order). Rudimentary ribs on the 20th vertebra were scored as lumbar ribs except when a rudimentary first rib was also present. In two cases a cervical rib was present in combination with the absence of ribs on the 18th vertebra; these cases were scored as a cervical rib and an absent 12th rib. In two cases a cervical rib was present in combination with ribs on the 20th vertebra; these cases were scored as a cervical rib and a lumbar rib.

Diagnosis of abnormalities and diseases

Standard autopsy reports were made by pathologists and filed in a national pathological archive (PALGA). We searched the reports for single and multiple congenital abnormalities and, if documented, cytogenetic abnormalities. Furthermore, we distinguished between minor and major congenital abnormalities following Merks et al.(2003) for external and skeletal abnormalities and Lancaster and Pedisich (1995) for further abnormalities. We opted for a classification in terms of minor/major and single/multiple abnormalities, because it reflects our expectations on the strength of the deleterious effects of these abnormalities. The condition of the environment (e.g. the mother) and the condition of the individual both influence the chance of a premature

death. Hence, the strength of the deleterious effects of the abnormalities provides information on the selective disadvantage. Examples of minor congenital abnormalities are hypertrophy of the heart, spleen, liver or lungs, supernumerary phalanges, club foot, hydrops foetalis (edema of the whole body), ventricular septal defect (defect in the wall that separates the left and right ventriculi of the heart), atrial septum defect (defect in the wall that separates the left and right atria of the heart), patent foramen ovale (persistence of the fetal opening between left and right atria), patent truncus arteriosus (persistence of the fetal structure between the left pulmonary artery and the descending aorta), omphalocele (intestines and other abdominal organs protrude into the base of the umbilical cord) and slight facial dysmorphologies. Examples of major congenital abnormalities are the absence of one or both kidneys, anencephaly and other failures of neural tube closure (Figure 2), absence of the corpus callosum in the brain, cyclops, cleft lip/palate, tracheo-esophageal fistula (abnormal passage between trachea and oesophagus), atresia of the aorta, dextroposition of the heart, monoventricular heart, mono-atrial heart, various chondrodysplasias (abnormally short and deformed limbs) and sirenomelia (abnormal development of the caudal part of the body with different degrees of fusion of the legs). In addition we considered dysmaturity and embryonal tumours to be major abnormalities and prematurity a minor abnormality.

Statistical analysis

We used generalized linear modelling techniques for analysis (Agresti 2002). Probabilities of occurrence of vertebral variations were studied by means of logistic regression, associations were investigated using log-linear analysis. In the log-linear analyses all associations between explanatory variables (types of congenital anomalies) were fitted in the models, in addition to associations of vertebral variations with congenital anomalies. This approach was followed because an analysis based on marginal totals of the contingency table alone can easily lead to incorrect conclusions

about an association, i.e., an association between vertebral variations and one classification variable of congenital abnormalities can reverse sign when the occurrences in different groups (categories of other classification variables of congenital anomalies) are pooled (Simpson's paradox, Simpson 1951). In all cases, we checked for overdispersion of the data relative to the model. We used backward model selection (from elaborate to simpler) to compare models and test hypotheses. Model comparison was done using likelihood ratio tests for nested models, and AIC (Akaike Information criterion, Akaike 1973) for non-nested models. Only test statistics from the selected logistic regression models and log-linear models are presented. Throughout a significance level of 95% was used. Confidence intervals reported for probabilities of occurrence are calculated from logistic regression models (Venables and Ripley 2002).

Incidence of vertebral variations in the general adult population

In order to estimate the prevalence of cervical, rudimentary first, absent or rudimentary twelfth or lumbar ribs in the general adult population, we analyzed a number of existing datasets, chosen such that datasets of diseased populations were excluded (see Table 1). We fitted a binomial regression model for the incidence of each variation, with *type* of study (radiographic, dried skeletons) and *study* as factors. This model was simplified parameter-by-parameter until all remaining effects were significantly different from zero. In this manner we lumped studies where differences in incidence were non-significant, for instance because of relatively low sample sizes of vertebral variations. The estimates reported for the calculation of the selective disadvantage are the maximum among the predicted values from the selected model for cervical ribs and the minimum one for absent or rudimentary twelfth ribs.

Selection against vertebral variations

In order to arrive at estimates of selection pressures we calculated the survival pattern of individuals with or without a cervical rib and an absent or rudimentary 12th rib, starting

from clinically recognized pregnancies. Our calculations are based on an age-structured population model with four age classes (numbered zero to three) and calculations are presented in Tables 2 and 3. We have assumed for the general population in Western Europe that the probability of death is $\mu_0 = 0.15$ in the age interval before birth, $\mu_1 = 0.01$ of neonatal mortality from birth to one year of age, approximately zero mortality in the interval from age one to maturity, $\mu_2 = 0$ (Regan et al. 1989; De Galan-Roosen et al. 1998; Cartlidge and Stewart 1995; Eurocat Report 1997, 1999; Saraiya et al. 1999; Nolte et al. 2000; Van Duin 2002). Adults must die, $\mu_3 = 1$. The calculations proceed as follows. Survival up to age S_i in the general population is equal to $S_i = (1 - \mu_{i-1})S_{i-1}$, with $S_0 = 1$. The prevalence of ribs among deaths that occurred in age class i are denoted p_i. We obtained those prevalence parameters from our data analysis. The prevalence P_i among the group that is alive in age i is equal to the prevalence among those that die from that class and those that survive into the next age, weighted by the probabilities of dying, resp surviving. This gives expression $P_i = \mu_i p_i + (1 - \mu_i)P_{i+1}$. From this prevalence P_i and survival S_i one can calculate the survival probability of individuals with a rib up to age i as $S_{Ri} = P_i S_i / P_0$. The survival probability of individuals without a rib variation becomes $S_{NORi} = (1 - P_i)S_i / (1 - P_0)$.

RESULTS

The incidence of cervical ribs (a rib on the seventh cervical vertebra, Fig.1) in deceased fetuses and infants was extremely high relative to the incidence in adults estimated from dry skeletons and radiographs (54.8 (confidence interval 50.5-59.0) % versus 1.1 (0.3-2.0) % in the study on adults with the highest incidence, see Table 1). Using backward model selection in a logistic multiple regression, we found no significant effects of age

at death, weight, year nor sex on the prevalence of cervical ribs. The absence of an age

effect (slope 0.0001 (s.e. 0.0003); $\chi^2 = 0.18$, p = 0.67) is evidence that cervical ribs don't

disappear later in development. We had no cases of completely absent first ribs.

Rudimentary first ribs were rare (2.1 (1.1-3.6) % versus 0.5 (0.3-0.9) % in the reference

populations). The incidence of absent or rudimentary twelfth thoracic ribs is 5.6 (3.8-

7.7) % (versus 3.0 (2.2-4.0) % in the reference populations), of lumbar ribs 1.3 (0.58-

2.6) % (versus 8.8 (6.6-11.3) % in the reference populations). These incidences showed

no age-dependence either. Possibly, the incidence of lumbar ribs in deceased fetuses and

infants is somewhat underestimated due to a delay in ossification of lumbar ribs

compared to thoracic ribs. There was no significant difference between the incidences

of fetuses from abortions for medical reasons and fetuses that died naturally ($\chi^2 = 0.001$,

p > 0.9).

Selection against cervical Ribs

We calculated the average selective disadvantage, combining our results with those on

frequencies of cervical ribs in adults and common survival patterns of human

populations. Using the lower confidence limit for the selective difference (the minimal

reasonable estimate of selective disadvantage), we found that at least 78% of the

individuals in recognized pregnancies with a cervical rib die before birth and 83%

before one year (Table 2). For individuals without cervical ribs 9% are dead by one year

of age. The relative probability of survival of individuals with cervical ribs to one year

of age, compared to individuals without, is at most 19%. If we assume that all

individuals for which radiographs were difficult to assess have no cervical ribs, then at

least 76% of the individuals with a cervical rib would die before birth and 81% before

one year. The relative probability of survival of individuals with cervical ribs to one

year of age, compared to individuals without, would increase to 21%. One can conclude that our conclusions are at most little biased by the missing observations.

Weaker Selection against a Change at the thoraco-lumbar Boundary

We repeated the above calculations for absent and rudimentary twelfth thoracic ribs, using the upper confidence limit for the selective difference (the *maximal* reasonable estimate of selective disadvantage). We found that at most 38% of the individuals of recognized pregnancies with an absent or rudimentary twelfth rib die before birth and 40% before age one (Table 3). Hence, the relative probability of survival of individuals with absent or rudimentary twelfth ribs compared to individuals without this variation is 73%. Assuming that all four individuals for which radiographs were difficult to assess at the thoraco-lumbar boundary had absent or rudimentary 12th thoracic ribs, this relative probability decreases to 70%. Thus, this conservative calculation indicates that selection against a change at the thoraco-lumbar boundary is much weaker than that against a change at the cervico-thoracic one.

Association of cervical ribs with congenital abnormalities

The incidence of major anomalies in deceased fetuses and infants was 66.7 (62.4 – 70.9) % . In 64.2 (59.7 – 68.5) % of the cases, multiple abnormalities were present. As a next step in our analysis, we investigated whether an association with congenital abnormalities could possibly explain the observed early deaths. The incidence of cervical ribs was indeed found to be positively associated either with multiple or with major congenital anomalies (both $P[>\chi^2] = 0.001$, see also the raw data in Table 4). A model including an effect of major anomalies fits the data better than one including

multiple anomalies, based on the AIC (AIC major anomalies 611.1, AIC multiple anomalies 613.9) The highest incidence of cervical ribs (63.6% (57.4-69.4%), Fig.3) was found in fetuses and infants with both multiple and major abnormalities. This significant association between cervical ribs and the most deleterious class of abnormalities, and the parallel association between cervical ribs and the total number of mildly or strongly deleterious effects supports our hypothesis that the presence of cervical ribs is selectively disadvantageous because of associated negative pleiotropic effects.

Next to a positive association with major or multiple anomalies, no further significant, or higher-order, associations of variables with the incidence of cervical vertebrae were found. We found no effects of age or weight on the association and no association with sex. No other vertebral variations were significantly associated with any of the mentioned variables. It is of some interest to note that the few cases of rudimentary first ribs were always associated with major congenital abnormalities, even though the incidence was too low for reaching significance.

Unilateral ribs

In 22.8% (18.0 - 28.0 %) of all cervical rib cases the cervical rib occurred only on one side. In our dataset left-sided unilateral cervical ribs occurred approximately twice as often as right-sided ones (39 vs. 22, χ^2-test, p = 0.02). In the log-linear analysis, the association between major or multiple congenital abnormalities and cervical ribs was not different for unilateral and bilateral cervical ribs (e.g., difference unilateral×major 0.17 (s.e. 0.39), χ^2 = 0.1, P[>χ^2] = 0.75). In addition, we found one unilateral

rudimentary first rib and one unilateral lumbar rib. In 9.4% (2.4 - 22.5%) of absent or rudimentary twelfth ribs, these were unilateral.

Small and large Scale Mutations

Only for a limited set of cases we could get access to cytogenetic analyses. The incidence of cervical ribs in cases of single-gene disorders (e.g. autosomal recessive polycystic kidney disease, cystic fibrosis, osteogenesis imperfecta, campomele dysplasia, chondrodysplasia punctata, split hand/split foot syndrome, Werdnig Hoffmann muscular atropy, Arthrogryposis multiplex; 66.5% (46.8 - 83.1%), n = 24) is approximately the same (difference test, $\chi^2 = 0.004$, p > 0.9) as that for cases with mutations that affect a large part of the genome, such as when a chromosome is absent (monosomy) or supernumerary (trisomy; 63.0% (49.7 - 75.0%) in 54 confirmed cases of trisomy, monosomy and triploidy (difference test, $\chi^2 = 0.004$, p > 0.9). This indicates that a strong association between cervical ribs and congenital abnormalities is not only found when many genes are missing or defect and the association may be due to an interaction of independent events, but also when only one gene is affected and the cervical ribs and other abnormalities are, thus, all pleiotropic effects of that gene. An other conclusion is that many different mutations can lead to cervical ribs, although generally in combination with deleterious pleiotropic effects.

Homeotic transformations of all thoracic vertebrae

We found that 26.4% (21.6 - 31.8%) of cervical ribs appear to be accompanied by a homeotic shift of all thoracic vertebrae, indicated by the full, or partial, loss of a rib on the most posterior thoracic vertebra (a posteriorization of the identity of a thoracic into a

lumbar vertebra, see paragraph Homeotic transformations in the Discussion). Of the rudimentary first ribs (an anteriorization of the first thoracic vertebra) 45.5% (19.4 - 73.5%) were associated with a rudimentary rib on the first lumbar vertebra (anteriorization), also pointing to a homeotic shift of all thoracic vertebrae and of the first lumbar vertebra.

DISCUSSION

We find an extremely high incidence of cervical ribs in deceased fetuses and infants compared to a low incidence in adults. High incidences in deceased fetuses and low incidences in adults have been reported before (Leboucq 1898; Rosenberg 1899; Bardeen 1904; Noback and Robertson 1951; Meyer 1978). This was never related to the constraint on the cervical vertebral number. To the contrary, as an explanation it has been proposed that cervical ribs may disappear by fusing with the vertebra after birth (Chernoff and Rogers 2004). This would imply that 98% of the cervical ribs disappear before birth (a decrease from 50% to 1%). However, traces of fusion of earlier bony extensions in normal adult seventh vertebrae have, as yet, never been documented. In addition, we found no effect of age on the incidence of cervical ribs accompanying foetal and infant deaths and deceased fetuses as well as deceased infants had cervical ribs in approx. half of the cases. We analysed only two children who died past the age of one (18 and 20 months), but both still had a cervical rib. Furthermore, although there is no good documentation of the incidence of cervical ribs in fetuses and children of the general population, the incidence of cervical ribs in the only study that we found of

healthy children (25,949 healthy, female child-relatives of Tuberculosis patients,

Menárguez Carretero and Campo Muňoz 1967) is well within the range found for adults

of the general population (0.5% for children versus 0.04-1.07% for adults, see Table 1).

Furthermore, the incidence of cervical ribs in populations of diseased children and

adults also appears to be very similar and varies between one and six percent (mainly

patients with infections and pulmonary diseases, Davis and King 1939; Schumacher et

al. 1992; Mercks et al. 2005 versus Coury and Delaporte 1954; Pionnier and Depraz

1956; Whittaker 1957; Erken 2002). These comparisons do not support a dramatic

decrease in incidence of cervical rib frequency during infanthood or childhood. A

higher incidence of cervical ribs in children was only found in children with embryonal

tumours (Schumacher et al. 1992; Mercks et al. 2005), an association which probably

provides further selection against cervical vertebrae (Galis 1999; Galis and Metz 2003).

Finally, half of the cervical rib cases in our dataset were not isolated, and were

accompanied by posteriorizations of the identity of one or more thoracic vertebrae. This

supports our assumption that cervical ribs in fetuses and infants do not indicate normal

development of a seventh cervical vertebra with a transiently present rib, but instead

represent a homeotic transformation of the seventh cervical vertebra into that of

adjacent, rib-bearing, first thoracic vertebra. We conclude, therefore, that the differences

between adult and prenatal incidence of cervical ribs are mainly due to early differential

mortality of individuals with a cervical rib.

Strong selection against Changes of the Number of cervical Vertebrae

Our data, thus, indicate very strong selection against changes in the number of cervical

vertebrae. The most frequent changes were bilateral and unilateral cervical ribs.

Rudimentary first ribs were surprisingly rare, but without exception associated with major abnormalities. It is possible that we underestimate the incidence of rudimentary first ribs if they are frequently associated with abnormalities that lead to a very early death, i.e. before the ribs ossify and we can detect them on radiographs. It is also possible that rudimentary first ribs are rarer. This needs further investigation.

We found that selection against changes at the thoraco-lumbar boundary is much weaker than that against a change at the cervico-thoracic one. The number of thoracic vertebrae indeed varies much more amongst mammals than the number of cervical vertebrae (Galis 1999; Narita and Kuratani 2005), attesting to a good agreement between the strength of selection and the apparent evolutionary constraints.

Selection against pleiotropic effects

It is highly improbable that the mere presence of cervical ribs would lead to fetal or infant deaths. Direct problems related to cervical ribs, compressions of nerves and arteries (thoracic outlet syndrome), usually only appear in adults (Makhoul and Machleder 1992; Roos 1996). Apparently, the constraint on the number of cervical vertebrae should mainly be sought in selection against congenital abnormalities associated with changes of this number (i.e., pleiotropic effects, Galis 1999). Our analysis is in agreement with earlier found associations of cervical ribs with abnormalities (Gladstone and Wakeley 1932; Adson and Coffey 1947; Keeling and Kjaer 1999; Steigenga et al. 2006) and with the absence in our dataset of such an association for rudimentary twelfth ribs. In addition, individuals with cervical ribs that survive infanthood experience on average more cancer than individuals without cervical ribs. There is a significant association of cervical ribs with specific pediatric cancers

(Schumacher et al. 1992; Mercks et al. 2005; see also Galis and Metz 2003). The multitude of deleterious pleiotropic effects dramatically limits the chances of individuals with cervical ribs to develop into viable adults.

Homeotic transformations

Homeotic transformations were first described by Bateson (1894) and are transformations of the identity of one structure into that of another. A well-known example is the transformation of the antennae of insects into legs as a result of antennapedia mutations (Abbott and Kaufman 1986). Cervical ribs appear to be partial, or complete, homeotic transformations of the seventh cervical vertebrae into rib-bearing thoracic vertebrae, i.e. a posteriorization of the identity. Fishel (1906) and Leboucq (1898) found that vertebrae with a cervical rib usually display more shape characteristics of thoracic vertebrae than the mere presence of a rib. In addition, Fishel (1906) and Oostra et al. (2005) conclude that in the majority of cases cervical ribs are not isolated events, but are accompanied by homeotic changes of several adjacent cervical and thoracic vertebrae. In agreement with their observation, we found that approximately one quarter of cervical ribs appear to be accompanied by a homeotic shift of all thoracic vertebrae. In addition, a similar proportion of cervical ribs is unilateral and these tend to be accompanied by a larger first thoracic rib on that side than on the contralateral side, indicating a partial homeotic change of the first thoracic vertebra into that of the more posterior second thoracic vertebra. Of the rudimentary first ribs (an anteriorization of the first thoracic vertebra) at least 20% appear to be associated with a homeotic shift of all thoracic vertebrae and of the first lumbar vertebra.

The *Hox* genes appear to be essential mediators of the anterior-posterior patterning of the presomitic mesoderm of the cervico-thoracic region and, hence, to be involved in homeotic changes of vertebral identity (Gaunt 1994; Burke et al. 1995, Cohn and Tickle 1999; Stern et al. 2006). The expression of *Hox* genes involved in this patterning is spatially and temporally co-linear and highly conserved (the zootype of Slack et al. 1993, see also de Rosa et al. 1999, Stern et al. 2004). Our data suggest that mutations with an effect on the conserved expression of these genes during the anterior-posterior patterning of the paraxial mesoderm may be common, but are strongly selected against.

Left-right asymmetry

In nearly a quarter of all cervical rib cases the cervical rib occurred only on one side, predominantly on the left side. Interestingly, this suggests that these cervical ribs are caused by a diminished coordination between the development of the left and right somites. Asymmetric left-right somite formation has recently been induced by deficient retinoic acid signaling in mice, chickens and zebrafishes (Vermot and Pourquié 2005; Kawakami et al. 2005; Vermot et al. 2005). Asymmetric somite formation was correlated with and presumably due to asymmetric somitic clock expression. In mice deficient signalling led to an acceleration in the development of the left somites compared to the right ones, with the asymmetry most prominent in the somites around the cervico-thoracic boundary (Vermot et al. 2005). A unilateral cervical rib in human fetuses and infants may similarly be the result of an acceleration of somitogenesis in the somites on the side of the rib (usually the left!) leading to a differential expression of the *Hox* genes that mediate anterior-posterior identity. In our dataset left-sided unilateral cervical ribs occurred approximately twice as frequently as right-sided ones. The left-

right asymmetries in vertebrae were never accompanied by other left-right asymmetries, such as asplenia, abnormal lung lobation and intestinal malrotation. This pattern is in full agreement with the independence found in mice and zebrafishes for the left-right symmetric development of the somites and the left-right asymmetry of internal organs (Vermot and Pourquié 2005; Kawakami et al. 2005).

Interactivity during the early patterning of the anterior-posterior axis

The determination of the position of the cervico-thoracic boundary mediated by the *Hox* genes forms part of the early anterior-posterior patterning of the presomitic mesoderm during the early neurula stage (e.g. Gaunt 1994; Burke et al. 1995; Cohn and Tickle 1999; Chernoff and Rogers 2004; Stern et al. 2006). The fact that more than half of all fetal and infant deaths come with cervical ribs emphasizes once again the vulnerability of the early organogenesis, or neurula stage (Galis and Metz 2003). The association of cervical ribs with multiple and with major abnormalities in other parts of the body (see Table 4) moreover points at the interaction of the early anterior-posterior patterning of the paraxial mesoderm with many other patterning processes and many morphogenetic processes. Corroboration for this viewpoint is, firstly, provided by grafting experiments in which the anterior-posterior position of paraxial mesoderm was changed and which led to changes in a) the anterior-posterior patterning of the adjacent neuroepithelium (Bel-Vialar 2002; see also Grapin-Botton et al. 1997; Ensini et al. 1998), b) the timing of the migration of neural crest cells (Sela-Donenfeld and Kalcheim 2000) and c) the initiation and outgrowth of the limbs (Saito et al. 2006). Secondly, this viewpoint is corroborated by experiments in which two processes that are involved in the determination of the anterior-posterior patterning of paraxial mesoderm were

manipulated: the opposing and antagonistic gradient of Fgfs, Wnts and RA and the oscillatory gene expression (somatic clock) in the paraxial mesoderm. These experiments have demonstrated couplings of the anterior-posterior patterning of paraxial mesoderm with morphogenetic processes such as proliferation and axial lengthening (Dubrulle et al. 2001; Dubrulle and Pourquié 2004), somitogenesis (Zakany et al. 2001; Dubrulle et al. 2001), convergent extension (Ninomiya et al. 2004, see also Mathis et al. 2001) and cell migration (Yang et al. 2001), as well as with patterning along the other embryonic axes, i.e. left-right and midline patterning (Raya et al. 2004, Krebs et al. 2003 see also Yamamoto et al. 2003 and Latimer et al. 2002) and dorso-ventral patterning (Diez del corral et al. 2003). There is thus a wealth of data supporting the strong coordination of the patterning of the three embryonic axes in the three adjacent germ-layers with a central role of the mesoderm in this process (see also Kumar et al. 2003) and, additionally, there is strong support for a coupling between patterning and morphogenetic processes.

The determination of the thoraco-lumbar boundary occurs later than that of the cervico-thoracic boundary. The lower frequency of shifts of this boundary suggests that this determination is less vulnerable. A lower interactivity is suggested by the absence of a significant association between shifts of this boundary and congenital abnormalities. The supposedly lower interactivity and vulnerability of this later stage at which the number of thoracic vertebrae is determined is a potential explanation for the weaker evolutionary constraint on changes of the number of thoracic vertebrae.

Modularity, stabilizing Selection and Conservation

The number of cervical vertebrae is determined during the early organogenesis stage (also referred to as phylotypic stage) that is itself strongly conserved in mammals (for a review see Hall 1999, but see Richardson et al. 1997). Sander (1983) and Raff (1994) proposed that the high interactivity between modules is the major cause of conservation in this stage. This high interactivity causes mutations to have negative pleiotropic effects that become amplified as development proceeds. Conservation is a consequence of consistently strong stabilizing selection against mutations via their pleiotropic effects. We earlier found support for the validity of this hypothesis in an analysis of teratological studies in rodents (Galis and Metz 2001). We found that chemical and other disturbances of this stage (phenocopies of mutations) lead to a considerably higher mortality than disturbances of earlier and later developmental stages. From the pattern of multiple induced abnormalities (pleiotropic effects), we concluded that it is the high interactivity and low effective modularity that is the root cause of the vulnerability of the stage: a particular, potentially useful change almost always will induce lethality even before the organism is exposed to ecological selection.

During early organogenesis the organ primordia make their first appearance. Hence the evolutionary conservation of early organogenesis in mammals may well be implicated in the conservation of the number of repeated organs (eyes, ears, vertebrae, limbs and digits; for the latter, see e.g. Galis et al. 2002). The present study indeed strongly suggests that during organogenesis the high interactivity and low modularity of the patterning of the anterior-posterior axis in the cervical paraxial mesoderm appears to be the root cause for the selective early deaths of humans with a changed number of

cervical vertebrae. Mutations that change the number of cervical vertebrae almost always appear to have many negative pleiotropic effects that cause mortality in fetuses and infants.

Low effective modularity, pleiotropic effects and strong selection, thus, appear to be important for both the conservation of the number of cervical vertebrae, as well as that of the entire stage during which this is determined. This implies that the evolutionary constraint on changes of early organogenesis, including changes of the number of cervical vertebrae, derives at least in part from a developmental constraint (Maynard Smith et al. 1985; Amundsen 1994; Schwenk and Wagner 2003; Arthur 2004; Amundson 2005). The developmental constraint is not a constraint in the sense that there is no production of mutational variation for the number of cervical vertebrae (i.e., a constraint of the 'forbidden morphologies' type), but in the sense that the development of this variation is accompanied, sheer unavoidably, by the development of variation in other traits that dramatically reduces the fitness. We are, thus, dealing with so-called pleiotropic constraints biasing the response to selection to an extent that it leads to evolutionary stasis (Hansen and Houle 2004).

Opitz et al. (1987) have argued that humans continue to be exposed to strong internal (i.e., not ecologically mediated) selection, for instance selection against supernumerary digits, cyclopia and other abnormalities (88.9% and 97.7% dead at the time of birth). Our results extend their conclusion to changes in the number of cervical vertebrae. Thus, strong natural selection in humans still occurs prenatally and it appears to play a major role in the conservation of at least one aspect of our body plan. The strong selection against changes of early organogenesis in rodents (Galis and Metz 2003) and against supernumerary digits in humans (Opitz et al. 1987), implicates internal selection to be of more general importance for the conservation of our common body plan.

As a final conclusion we wish to draw the attention to the fact that changes of highly conserved traits of the body plan, such as the number of cervical vertebrae, may well be useful as indicators of medical risks (Steigenga et al. 2006).

ACKNOWLEDGEMENTS

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Table 1. Rib variation frequencies in the general population. Studies are of two types, either based on radiographies that were made for Tuberculosis-prevention, or based on collections of dried skeletons.

Type	Study	N.	N. with cervical rib (%)	N. with absent or rudimentary 1st rib (%)	N. with absent or rudimentary 12th rib (%)	N. with lumbar rib (%)
Rad.	Berner 1944	4333200	9182 (0.21%)	240 (0.006%)	n.a.	n.a.
Rad.	Henderson 1913	80000	31 (0.04%)	5 (0.006%)	n.a.	n.a.
Rad.	Crimm 1952	40000	68 (0.17%)	67 (0.17%)	n.a.	n.a.
Rad.	Crimm 1952	71877	116 (0.22%)	n.a.	n.a.	n.a.
Rad.	Steiner 1934	38105	19 (0.05%)	5 (0.01%)	n.a.	n.a.
Rad.	Southam & Bythal 1924	2000	9 (0.45%)	n.a.	n.a.	n.a.
Rad.	Sycamore 1944	2000	10 (0.5%)	10 (0.5%)	n.a.	n.a.
Skel.	Lanier 1944	559	6 (1.07%)	0 (0%)	20 (3.58%)	49 (8.77%)

Skel.	Fishel 1906	524	5 (0.95%)	n.a.	n.a.	n.a.
Skel.	Topinard 1877	350	2 (0.57%)	0 (0%)	5 (1.43%)	2 (0.006%)
Skel.	Paterson 1893	132	0 (0%)	0 (0%)	2 (1.52%)	1 (0.76%)
Skel.	Bianchi 1894	130	0 (0%)	0 (0%)	5	3
Skel.	Staderini 1894	100	1 (1%)	0 (0%)	6 (6%)	2 (2%)
Skel.	Steinbach 1889	83	0 (0%)	0 (0%)	1 (1.20%)	3 (3.61%)
Skel.	Bardeen 1904	70	0 (0%)	0 (0%)	4 (5.71%	3 (4.29%)
Skel.	Ancel & Sancert 1902	43	0 (0%)	0 (0%)	1 (2.33%)	3 (6.98%)

Abbreviations: Rad.-radiographs, Skel.-dried skeletons, n.a.-not available.

Table 2. Selection against cervical ribs.

Age Class i	μ_i	Survival up to age i	Prevalence among those that died p_i	Prevalence P_i	Relative survival up to age i with cervical ribs S_{Ri}	Relative survival up to age i without cervical ribs S_{NORi}
0	0.15	1	0.505	0.097	1	1
1	0.01	0.85	0.505	0.025	0.218	0.918
2	0	0.8415	0.020	0.020	0.174	0.913
3	1	0.8415	0.020	0.020	0.174	0.913

Table 3. Selection against absent or reduced twelfth thoracic ribs

Age Class i	μ_i	Survival up to age i	Prevalence among those that died p_i	Prevalence P_i	Relative survival up to age i with 12th thoracal ribs S_{Ri}	Relative survival up to age i without 12th thoracal ribs S_{NORi}
0	0.15	1	0.077	0.031	1	1
1	0.01	0.85	0.077	0.022	0.624	0.857
2	0	0.8415	0.022	0.022	0.603	0.849
3	1	0.8415	0.022	0.022	0.603	0.849

Table 4. Frequent congenital abnormalities in deceased human fetuses and infants (> 10 cases) and associated changes in the number of cervical vertebrae.

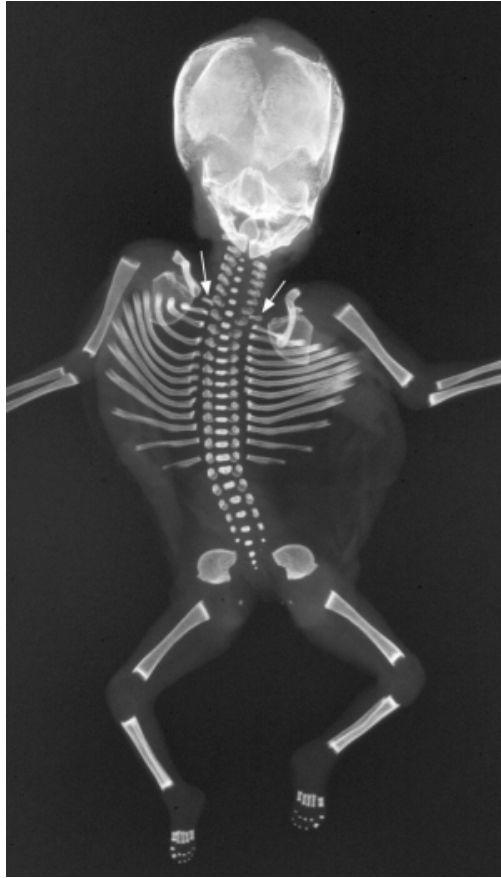| Congenital abnormality	N. of cases	N. with cervical rib (%)	N with absent or rudimentary first rib (%)	N with aberrant number of cervical vertebrae (%)
Cleft lip/palate	12	6 (50%)	3 (25%)	9 (75%)
Horseshoe kidney	10	8 (80%)	1 (10%)	9 (90%)
Bleeding disorders	98	68 (69.4%)	1 (1%)	70 (70.4%)
Oligo/polydactyly	17	9 (52.9%)	4 (23.5%)	13 (76.4%)
Spina bifida	10	4 (40%)	1 (10%)	5 (50%)
Aberrant Arteria Subclavia dextra	22	18 (81.8%)	0 (0%)	18 (81.8%)
Ventricular septum defect	31	17 (54.8%)	8 (25.9%)	25 (80.7%)
Transfusion syndrome	14	8 (57.1)	0 (0%)	8 (57.1%)
Left-right disorders	21	15 (71.4%)	1 (4.8%)	16 (76.2%)
Bilateral kidney agenesis	10	6 (60%)	1 (10%)	7 (70%)
Spina bifida	11	5 (45.5%)	1 (9.1%)	6 (54.6%)
Anal atresia	11	6 (54.5%)	3 (27.3%)	9 (81.8%)
Hydrops foetalis	22	10 (45.5%)	1 (4.5%)	11 (50%)
Dysmaturity	59	33 (55.9%)	0 (0%)	33 (55.9%)
Prematurity	68	39 (57.4%)	2 (2.9%)	41 (60.3%)

Figure 1. Radiograph showing left and right rudimentary cervical ribs in a male fetus of 16 weeks of gestation. Arrows indicate cervical ribs. To enhance the visibility of the ossifications, the fetus has been stained with AgNO3. Institute of Pathology, Division Photography, Free University Medical Center, Amsterdam.
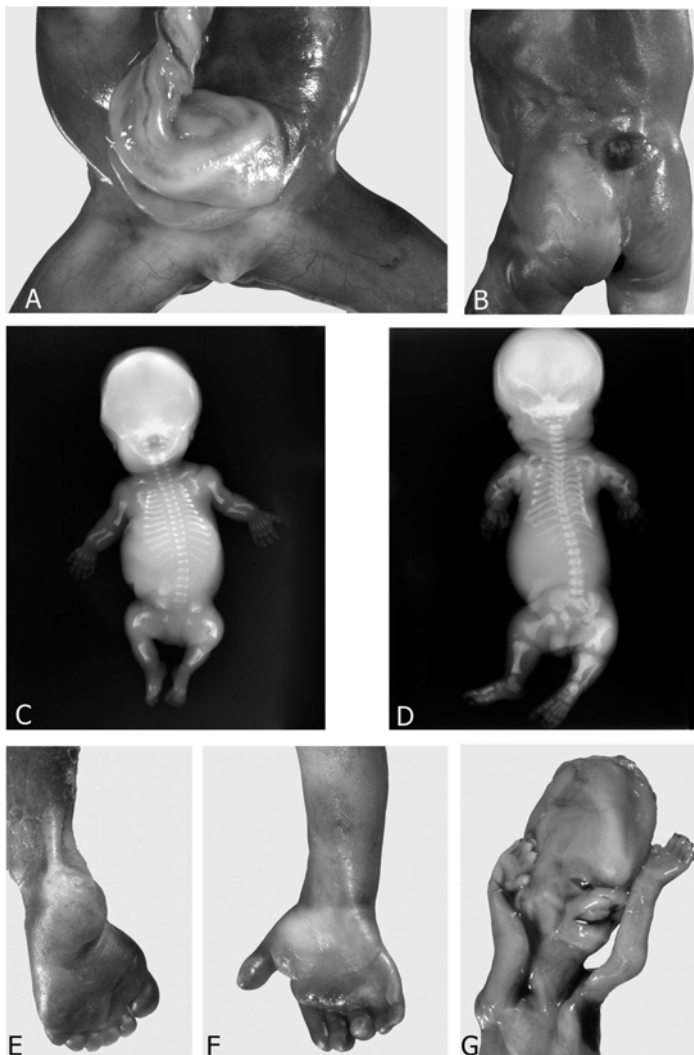
Figure 2. Photographs showing A) Omphalocele (an abdominal wall defect in which the abdominal contents protrude into the base of the umbilical cord) in a male fetus of 20 weeks gestation; B) Spina bifida (a failure of the closure of the neural tube in the lower back region) in a female fetus of 22 weeks of gestation; C) A skeletal dysplasia, thanatophoric dysplasia, with typically curved femurs in the shape of "French telephone receivers", in a female fetus of 22 weeks of gestation; D) Another skeletal dysplasia, Blomstrand dysplasia, characterized by an advanced skeletal maturation, in a male fetus of 32 weeks of gestation; E) and F) Post-axial polydactyly (extra digit posterior to the fifth digit) of foot and hand in a female fetus of 22 weeks of gestation (as part of short-rib-polydactyly syndrome); G) Median cleft lip in a male fetus of 15 weeks of gestation. Institute of Pathology, Division Photography, Free University Medical Center, Amsterdam.
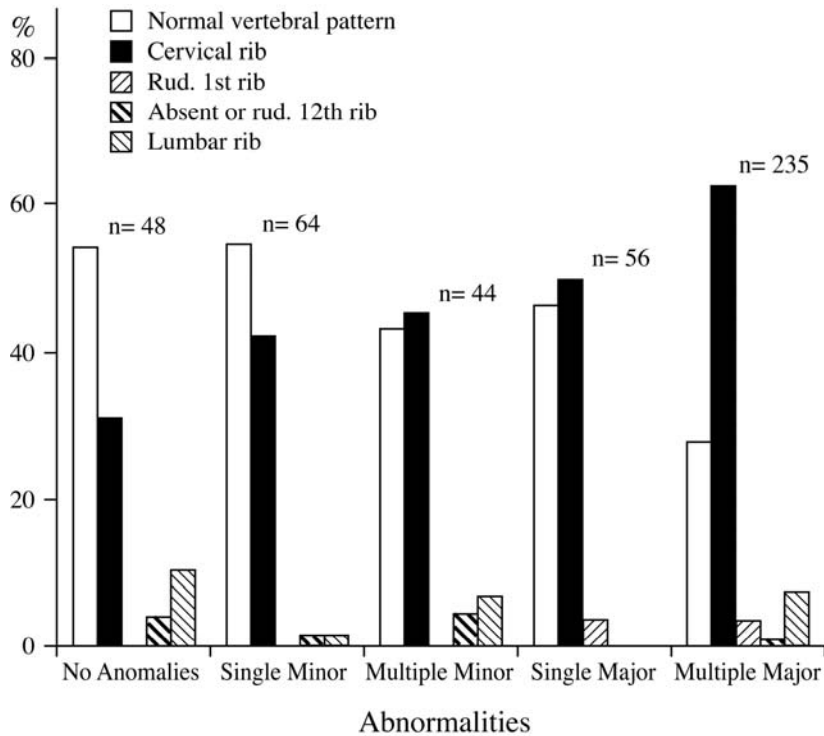
Figure 3. Graph showing the prevalence of cervical ribs, rudimentary first ribs, rudimentary or absent twelfth ribs and lumbar ribs in foetal and infant deaths with resp. no, single minor, single major, multiple minor and multiple major abnormalities. The incidence of cervical ribs increases with the number and severity of the abnormalities. Four cases had two vertebral variations, these were scored in both categories: two cases with a cervical and a lumbar rib, one in the single minor and one in the multiple major group; two cases with cervical ribs and absent 12th ribs (11 ribs including the cervical rib), both in the multiple major abnormalities group.