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## Higher limb asymmetry in deceased human fetuses and infants with aneuploidy

## SUBJECT AREAS:

DEVELOPMENTAL  
BIOLOGY

ZOOLOGY

ECOLOGY

EVOLUTION

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16 December 2013Published  
15 January 2014Correspondence and  
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**Aneuploidies cause gene-dosage imbalances that presumably result in a generalized decreased developmental homeostasis, which is expected to be detectable through an increase in fluctuating asymmetry (FA) of bilateral symmetric traits. However, support for the link between aneuploidy and FA is currently limited and no comparisons among different aneuploidies have been made. Here, we study FA in deceased human fetuses and infants from a 20-year hospital collection. Mean FA of limb bones was compared among groups of aneuploidies with different prenatal and postnatal survival chances and two reference groups (normal karyogram or no congenital anomalies). Limb asymmetry was 1.5 times higher for aneuploid cases with generally very short life expectancies (trisomy 13, trisomy 18, monosomy X, triploidy) than for trisomy 21 patients and both reference groups with higher life expectancies. Thus, FA levels are highest in groups for which developmental disturbances have been highest. Our results show a significant relationship between fluctuating asymmetry, human genetic disorders and severity of the associated abnormalities.**

In humans, many of the visceral organs are asymmetrically organized. For instance, the lung has three lobes on the right side and two on the left side, liver and gallbladder are positioned on the right side, stomach and spleen on the left side, and the apex of the heart points to the left<sup>1</sup>. By contrast, external traits such as limbs or facial structures appear symmetrical at first sight, and come as two mirror-image copies (bilateral symmetry). Since such traits can be considered as replicas of the same developmental events, they should develop identically mirrored under ideal conditions. Nevertheless, small perturbations of developmental processes cause development to deviate from its expected pathway, and since these processes act locally the effects of perturbations will accumulate in left and right sides separately<sup>2,3</sup>. This causes small amounts of asymmetry that are random with respect to side and therefore termed fluctuating asymmetry (FA).

FA is considered to be a measure of developmental instability (DI). DI reflects the inability of an organism to buffer its development against random perturbations (referred to as developmental noise) due to environmental or genetic stress<sup>3,4</sup>. For several decades it has been recognized that developing organisms have the capacity to withstand (to a certain extent) perturbations of various origin (i.e. developmental buffering or developmental homeostasis) (e.g.<sup>5–7</sup>). However, increased levels of disturbances cause a breakdown of the buffering capacity which leads to an increase in DI and thus also in measurable FA. Because of its ability to reflect DI, FA is thought to covary negatively with indices of fitness or health (reviewed in<sup>8–11</sup>), although more recent studies show that results are heterogeneous<sup>3,12</sup>. In particular in humans, it has been proposed that FA is correlated with and could therefore serve as risk marker for disorders of developmental origin, such as trisomy and genetic syndromes<sup>13</sup>. However, support is currently limited since only few studies found an association between FA and different kinds of developmental disorders<sup>14–20</sup>, while it was absent or inconsistent in others (e.g.<sup>21,22</sup>). Furthermore, the majority of the above-mentioned studies reporting an association (four out of seven) have been conducted in patients with trisomy 21 (Down syndrome), which showed augmented FA of craniofacial traits<sup>14–16,20</sup>. More studies are clearly needed that examine FA in relation to different kinds of aneuploidies and genetic defects to obtain a better understanding of the association between FA, DI and developmental disorders in humans.

It has been suggested that trisomy 21 may result in amplified developmental instability because the gene-dosage imbalance caused by the extra genetic material of chromosome 21 results in a generalized genetic



imbalance that decreases developmental buffering<sup>23</sup>. The imbalance (or abnormal gene copy number) alters the transcript level for certain genes compared to other genes and this may affect complex gene-regulatory processes and thereby alter development. Similarly, it is possible that aneuploidy in general results in a decrease of developmental buffering. In line with this possibility, Dunlap, et al.<sup>24</sup> found altered limb tissue development in trisomic 13, 18 and 21 deceased human fetuses, which occurred on the left side, right side, or bilateral. Dissections revealed absences, supernumeries and morphological anomalies of the neuromuscular phenotype<sup>24</sup>, however limb asymmetry was not examined. Furthermore, the effects of chromosomal number changes on FA, other than trisomy 21, have not been studied. Only aneuploidy fetuses with trisomy 13, 18 and 21, triploidy, monosomy X (Turner syndrome) and XYY (Klinefelter) are able to grow sufficiently (and thus allow FA measurements). Here, for the first time we study FA in deceased human fetuses and infants with several numerical chromosomal changes from a 20-year hospital collection.

In this study, we specifically aim to determine whether (1) FA increases with the presence of numerical chromosomal aberrations; (2) the level of FA differs between different kinds of aneuploidies; and (3) FA levels reflect the severity of the aneuploidy. We hypothesize that fetuses suffering complete aneuploidy with very limited or no postnatal survival rates have higher FA than fetuses with aneuploidies that on average have longer survival times, because we assume development to be more disturbed. FA is determined for the limb bones (ulna, radius, femur, tibia, fibula, digit 2 and digit 4) of human fetuses and infants using radiographs. Whereas previous studies focused mainly on single craniofacial traits, we determined FA of several limb bones because a recent meta-analysis indicated that using composite measures of multiple body traits yields more statistical power<sup>25</sup>. Furthermore, the advantage of using fetuses and young infants for limb FA is that traits are largely unaffected by mechanical loading, whereas in adult bone structures asymmetries may be the result of bone remodeling during growth<sup>26,27</sup>.

To be able to test for differences in FA between individuals with chromosomal number changes with dissimilar survival chances, we studied fetuses and infants that died in the VU University Medical Center (VUMC) in Amsterdam (The Netherlands) between 1990 and 2009. Fetuses with aneuploidies were classified to reflect the severity of the condition; in other words to reflect the survival chance of the patients. In general, aneuploidy has serious, often lethal consequences, with the exact consequences depending on how rich in genes the affected chromosomes are<sup>28</sup>. Based on the life expectancies described in the literature, the most commonly occurring aneuploidies in our dataset were classified into three groups. All studied fetuses and infants died prenatally or early in postnatal life and many suffered from minor and/or major congenital anomalies (for examples see<sup>22,29</sup>). Therefore, as a reference group, we used fetuses and infants without (observable) congenital abnormalities (group 0 – no congenital abnormalities) that were mainly aborted for maternal reasons, or with a normal karyogram (group 0 – normal karyogram) but with the possibility of having minor and/or major congenital abnormalities. The two reference groups are independent and both are expected to have a more normal development than individuals with aneuploidies. Assuming our hypothesis that DI shows a positive relationship with increasing severity of chromosomal abnormalities, we expect FA to be lower in these reference groups. Group 1 is restricted to fetuses and infants with trisomy 21 (Down syndrome). Patients with trisomy 21 have a relatively moderate postnatal survival prognosis with the help of medical care<sup>30</sup>, although pregnancies are liable to spontaneous abortion<sup>31</sup>. Considering the presence of extra chromosomal material, but also the relatively good survival prognosis compared to other studied aneuploidies, FA is expected to be moderately elevated in this group compared to the two reference groups. Lastly, we grouped individuals with less frequently occurring

aneuploidies that are all associated with an overall poor survival prognosis (group 2: trisomy 13, trisomy 18, monosomy X, and triploidy). Indeed, it has been reported that they have a high chance of intrauterine death<sup>32–35</sup> and a very short postnatal life span<sup>36–42</sup>. We therefore assume that development has been most severely disturbed in cases of group 2, which we hypothesize to be observable through an augmented level of FA compared to groups 0 and 1. Results of the comparison of limb asymmetry among these groups are reported using the FA averaged across the seven studied traits to obtain a single value for each individual (referred to as mean FA).

## Results

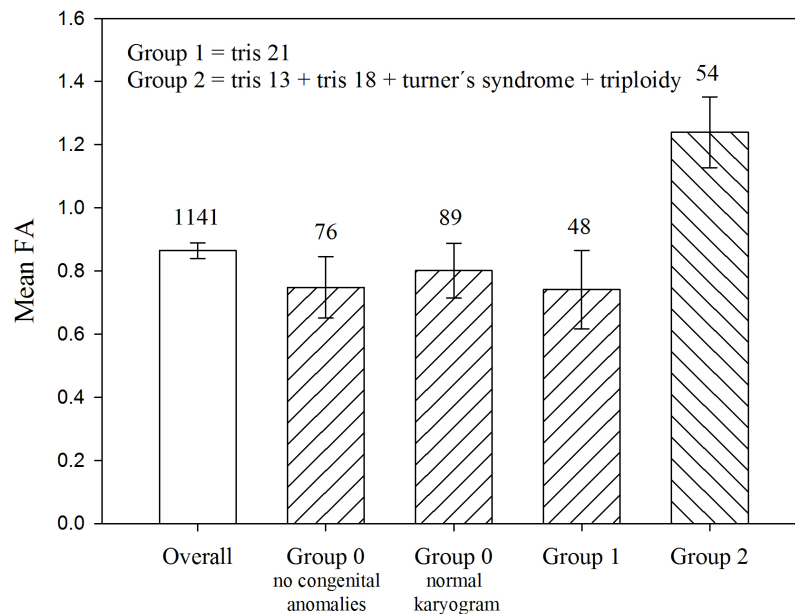
Mean FA differed significantly among the three groups of aneuploidies, both when using individuals without congenital anomalies ( $N = 76$ ) ( $F_{2, 163} = 5.71$ ,  $P = 0.004$ ) and individuals with a normal karyogram ( $N = 89$ ) ( $F_{4, 182} = 5.48$ ,  $P = 0.005$ ) as reference group 0 (Fig. 1). Pair-wise comparisons showed no significant difference in mean FA between group 0 and group 1 ( $N = 48$ ) (all Tukey- $P > 0.98$ ). By contrast, both group 0 (all Tukey- $P < 0.01$ ) and group 1 (all Tukey- $P < 0.023$ ) had a significantly lower mean FA compared to group 2 ( $N = 53$ ). Mean FA was approximately 40% lower in group 0 and group 1 than in group 2 (Figure 1). Furthermore, this study confirmed the presence of a negative association between gestational age (log transformed to assure linearity and in hours) and mean FA (group 0 - no congenital abnormalities:  $F_{1, 163} = 6.12$ ,  $P = 0.014$ ; group 0 - normal karyogram:  $F_{1, 181} = 2.02$ ,  $P = 0.016$ ), as previously observed by Van Dongen et al.<sup>22</sup> and ten Broek et al.<sup>43</sup>. Differences in mean FA between males and females were not significant in both analyses (group 0 - no congenital abnormalities:  $F_{1, 163} = 1.50$ ,  $P = 0.22$ ; group 0 - normal karyogram:  $F_{1, 183} = 0.53$ ,  $P = 0.46$ ).

We then explored whether the aneuploidies that were treated together in group 2 showed similar levels of FA. Table 2 shows that the mean FA was comparable for individuals with trisomy 18, Turner's syndrome and triploidy. By contrast, the level of FA appeared to be only half as much for trisomy 13. We also tested whether the level of FA varied among cases with trisomy 21 that were electively aborted and the ones that resulted in a spontaneous fetal or infant death. The mean FA for individuals from electively aborted pregnancies was  $0.73 \pm 0.14$ , whereas the mean FA for individuals that suffered a spontaneous death was  $0.82 \pm 0.30$ . Pair-wise comparison confirmed that this difference was not significant ( $P = 0.79$ ).

## Discussion

Here, we used a unique dataset of deceased human fetuses and infants to examine differences in mean FA of the limbs bones between individuals without congenital abnormalities or with a normal karyotype (group 0), patients with trisomy 21 (group 1), and cases with more severe chromosomal number changes that have a very short prenatal and perinatal life expectancy (group 2: trisomy 13, trisomy 18, monosomy X and triploidy).

Fetuses with trisomy 21 (Down syndrome) did not show higher limb FA than fetuses with either a normal karyotype or without congenital anomalies. This is an unexpected result because a significant relationship between FA and Down syndrome has been reported by several studies focusing on FA of craniofacial traits<sup>14–16,20</sup>. A possible explanation for the lack of association in this study may be that limb development is better buffered against genetic insult due to extra genetic material on chromosome 21 than craniofacial development. In agreement with this hypothesis, individuals with Down syndrome typically exhibit various facial dysmorphologies, including epicanthic folds, macroglossia, and hypertelorism (e.g.<sup>44</sup>). However, shortening of the limbs also has been widely described as a feature of Down syndrome (e.g.<sup>45</sup>), suggesting that extra genetic material of chromosome 21 does interfere with normal limb development to some extent. An alternative explanation for our results may be that



**Figure 1 | Mean FA (not standardized) in human fetuses and infants for the three groups of chromosomal abnormalities (based on the estimates of the statistical model).** Also, the level of FA of the overall population is depicted for comparison. Values above the bars show the sample size of each group. Note that the sample size of the overall population does not equal the sum of study groups 0 to 2, since it also contains cases with an unknown karyogram and congenital anomalies. An analysis using pair-wise comparisons indicated significant differences between group 0 and 1 on the one hand and group 2 on the other hand.

(a)symmetry development of the face reflects disturbances at later, postnatal developmental stages than the (a)symmetrical development of the limb bones in our study. Unfortunately, we could not compare craniofacial and limb FA, nor examine potential long-term temporal effects, since our data are based on radiographs that do not allow accurate measurement of facial traits and the majority of cases died prenatally. Finally, although the individuals in our reference groups did not have congenital anomalies or had a normal karyogram, they all died prenatally or perinatally and can therefore not be treated as a surrogate of the healthy population. As such, it cannot be completely excluded that their premature death and associated stress resulted in higher FA compared to normally developing individuals, thereby lowering the power to detect potential differences among groups. However, since it is impossible and unethical to carry out FA measurements in utero, these selected cases should be considered as the best possible reference group available.

Fetuses with aneuploidies belonging to group 2 with a very short prenatal and perinatal life expectancy showed higher limb FA than cases with trisomy 21, without congenital anomalies, and with a normal karyotype. Specifically, the effects on FA were 1.5 times higher for fetuses with these severe aneuploidies (trisomy 13, trisomy 18, monosomy X and triploidy), compared to group 0 and 1. Such a pattern may reflect a causal relationship between FA and the severity

of developmental disturbance. When putting this result in Shapiro's framework of amplified developmental instability<sup>23</sup>, it appears that the buffering capacity of limb development is more vulnerable to the genetic insult or gene-dosage imbalance of aneuploidies of chromosome X, 13 or 18 than of chromosome 21. In fact, since chromosome 13 and 18 are larger and carry more genes than chromosome 21<sup>28</sup>, gene-dosage imbalance effects likely have stronger negative effects on the buffering capacity, which becomes evident through higher FA levels. In individuals with triploidy, the worst possible breakdown of developmental homeostasis can be expected since an extra copy of all chromosomes is present. To further test this hypothesis, it would be of interest to examine whether FA of triploidy subjects is higher than trisomy subjects. However, most triploid pregnancies are aborted during early gestation<sup>28</sup>, so that sample size of measurable cases is extremely low ( $N = 10$  in our 20-year hospital collection). Therefore, we included them in group 2. Importantly, the mean FA of the different aneuploidies included in group 2 was comparable (except for trisomy 13, see table 2), confirming that the observed effect was not solely driven by triploid cases. Furthermore, our results for group 2 provide support for Møller's hypothesis<sup>46</sup> that individuals with high levels of DI and FA would be eliminated early and thus be absent from the surviving population. The non-random elimination of offspring during development

**Table 1 | Levels of measurement error resulting from mounting and radiographing (ME-radiogr.), digitalizing the radiographs (ME-digit.), measuring each photograph (ME-meas.), the percentage of variation due to ME relative to real FA (% ME), and the levels of directional asymmetry (DA) of the studied limb bones. Variance components were multiplied by 1000**

Trait	Real FA	ME-radiogr.	ME-digit.	ME-meas.	%ME	DA
Femur	2.18	0.79	0.10	0.19	33	$X^2 = 38.95$ , $df = 1$ , $P < 0.01$
Tibia	4.95	0.36	0.08	0.08	9	$X^2 = 0.00$ , $df = 1$ , $P = 0.99$
Fibula	2.75	1.16	0.05	0.10	32	$X^2 = 2.96$ , $df = 1$ , $P = 0.09$
Radius	1.17	0.51	0.29	0.14	45	$X^2 = 2.97$ , $df = 1$ , $P = 0.09$
Ulna	1.43	0.71	0.00	0.24	40	$X^2 = 4.03$ , $df = 1$ , $P = 0.04$
Digit 2	0.60	0.35	0.00	0.00	37	$X^2 = 0.93$ , $df = 1$ , $P = 0.34$
Digit 4	1.52	1.41	0.00	0.03	49	$X^2 = 0.74$ , $df = 1$ , $P = 0.39$



**Table 2 | Overview of the mean FA (not standardized) for aneuploidies that are treated together in group 2**

Aneuploidy	mean FA	SE	N
Trisomy 13	0.57	0.26	10
Trisomy 18	1.43	0.17	24
Turner's syndrome	1.38	0.27	9
Triploidy	1.34	0.25	10

(referred to as developmental selection), has recently been demonstrated experimentally by an increase in developmental instability in dead compared to live fruit fly males experiencing thermal stress<sup>47</sup>. Here, we provide evidence for developmental selection on developmental instability in a non-experimental population. Indeed, cases from group 2 both have an extremely high chance of intrauterine death and a short perinatal life span (e.g.<sup>32–34</sup>) and have the highest FA compared to the reference groups. To put our results in the context of the literature on humans, we calculated the effect size for group 2 vs. the reference groups and compared it with effect sizes reported in a meta-analysis of Van Dongen and Gangestad<sup>25</sup>. It seems that our findings are situated in the range of the highest effect sizes found in the FA literature. While Van Dongen and Gangestad<sup>25</sup> reported an average effect size of 0.10 (SE = 0.02, 95% C.I.: 0.07–0.14) for studies reporting associations with fitness and health in humans, the effect sizes for group 2 vs. the control groups in this study were roughly 2.5 times higher and differed significantly for the overall average (group 2 vs. group 0 no anomalies: effect size = 0.28; 95% C.I.: 0.11–0.43,  $p = 0.01$ ; group 2 vs. group 0 normal karyotype: effect size = 0.25; 95% C.I.: 0.09–0.40,  $p = 0.02$ ).

In conclusion, we demonstrate that (1) FA is augmented in deceased fetuses and infants with severe types of chromosomal abnormalities; (2) FA levels vary between different types of aneuploidy; and (3) FA reflects the severity of disturbance during development. The premature (prenatal or perinatal) death of individuals with augmented limb asymmetry provides support for developmental selection indirectly or directly acting on developmental instability. Finally, the finding that in aneuploid individuals subtle variation exists between left and right limb bones, besides the known characterizing dysmorphologies, indicates that a gene-dosage imbalance may cause a general disturbance of bilateral symmetric development, but the medical implications of this subtle variation remain unknown.

## Methods

**Limb asymmetry.** Since 1980, all deceased fetuses and infants presented for medical examination at the VUMC, have been routinely radiographed both ventrally and laterally (23 mA, 70–90 kV, 4–12 sec, Agfa [Mortsel, Belgium] Gevaert D7DW Structurix films). To allow digital image analysis, we digitalized these radiographs first using a standard set-up with a Canon 300D digital camera positioned in a fixed distance from a glass plate, with flash underneath. The collection includes fetuses that were aborted for medical reasons (e.g. fatal abnormalities or chromosomal abnormalities). Radiographs that had insufficient resolution or where the limbs were not suitably positioned for measurement were excluded from data analysis. In total, we measured 1141 individuals (497 females, 628 males, and 16 individuals of unknown sex). The age of the fetuses and infants ranged between twelve weeks of gestation and two years of age. For each individual, we determined the size of the left and right ulna, radius, femur, tibia, fibula, digit 2 and digit 4 in Image J version 1.46<sup>48</sup>. Images were spatially calibrated from a scale present on a photograph. Limb bones were measured from the midpoint of the proximal end to the midpoint of the distal end of the bone. Digits were measured from the proximal end of the proximal phalanx to the distal end of the distal phalanx (see also<sup>23</sup>). All measurements were conducted by four investigators, who had no prior knowledge of the autopsy reports (however several congenital abnormalities appear on the radiographs). To compare the accuracy between the investigators, 31 fetuses were re-measured independently. Spearman correlation tests showed that the unsigned left-right differences were highly comparable between measurers (all  $P < 0.001$  and all  $r > 0.30$ ). In addition, for 147 individuals the entire procedure of positioning and making the radiograph was repeated, and these radiographs were also digitalized and measured as described above. For 49 individuals, a second independent digital picture was made from the radiograph and for 30 individuals the digital pictures were measured twice. A mixed

regression model was run to determine measurement error (ME) and directional asymmetry (DA)<sup>49</sup>. Three types of measurement error could be determined: first, on the individual level when repeated measurements were taken on the same individual; second, as a result of positioning the fetus based on repeated radiographs of the same individual; and third, by measuring different digitized photographs of the same radiograph. Results in Table 1 show that levels of ME were smaller than levels of FA for the studied traits. DA was analyzed using F-tests<sup>49</sup>. DA was absent for all studied traits, except for femur and ulna where we detected a significant effect for side (Table 1). However, FA measurements were corrected for ME and DA by calculating the unbiased FA using the mixed regression model of Van Dongen<sup>49</sup>.

**Chromosomal abnormalities.** We specifically searched for chromosomal abnormalities in the standard autopsy reports that are filed in a national pathological archive (PALGA; www.palga.nl). For statistical reasons, the analysis was restricted to the most commonly occurring chromosomal abnormalities in our dataset, i.e. trisomy 13, trisomy 18, trisomy 21, monosomy X, and triploidy. Cases with Klinefelter syndrome were excluded from the analyses because of the low sample size in the dataset ( $N = 3$ ). Also, individuals with mosaic chromosomal number changes (when known) were excluded because of the varying severity with the numbers of cells affected. Based on the life expectancies described in the literature, the most commonly occurring aneuploidies in our dataset were classified into the following three groups. For completeness, we also provide the FA of the overall population (including all cases in the database except group 1 and 2) in the figures, but this is not included in the analysis to prevent statistical inference.

**Group 0.** As a reference group, we used fetuses without (observable) congenital abnormalities. Their development probably followed a normal or near-normal trajectory up until their premature death, which was often induced for maternal reasons ( $N = 76$ ). We also performed the same analysis with all individuals with a normal karyogram ( $N = 89$ ) as a reference group. Unfortunately, there were only two individuals with a normal karyogram that were also classified as having no congenital anomalies. This was mainly because the majority of the individuals with no abnormalities were not additionally karyotyped. It should thus be noted that individuals with a normal karyotype may have minor and/or major congenital abnormalities (e.g. spina bifida, hypoplastic left heart complex, renal agenesis) and that individuals without congenital abnormalities may have abnormal karyotypes.

**Group 1.** Fetuses and infants in this group have been diagnosed with trisomy 21 (Down syndrome) ( $N = 48$ ). Pregnancies of Down syndrome fetuses are liable to spontaneous abortions. For instance, when measured between chorionic villus sampling (between eleven and thirteen weeks of gestation) and term, 43 percent of the pregnancies end in miscarriage or stillbirth<sup>51</sup>. Nevertheless, this also indicates that a considerable number of Down syndrome patients may survive postnatally (if not terminated after prenatal diagnosis). Indeed, during the first half of the twentieth century (when medical care was limited), life expectancy ranged between eight and twelve years of age<sup>50</sup>. Since that time, childhood survival of Down syndrome patients has much increased as a result of enhanced medical care, such that their life expectancy is currently estimated to be 58.6 years of age<sup>50</sup>.

Additionally for group 1, we distinguished the level of FA between the Down subjects that suffered from spontaneous fetal or infant death ( $N = 8$ ; four cases with fetal death, two stillborn cases and two cases that died postnatally) and the ones whose parents had chosen for elective abortion ( $N = 40$ ). It is possible that differences in FA exist since the latter group contains individuals that may have survived longer if the pregnancy was not terminated and spontaneous abortion may be biased towards the individuals with the most extreme DI<sup>16</sup>.

**Group 2.** This group includes fetuses and infants with trisomy 13 (Patau syndrome,  $N = 10$ ), trisomy 18 (Edwards syndrome,  $N = 24$ ), monosomy X (Turner's syndrome,  $N = 9$ ), and triploidy ( $N = 10$ ). These conditions are lethal since nearly all affected individuals die prenatally or shortly after birth.

For pregnancies diagnosed with trisomy 13 and 18, it is estimated that respectively 49 and 72 percent end in a miscarriage or stillbirth between 12 weeks of gestation and term<sup>52</sup>. Furthermore, the median survival time of liveborn infants varies between 2.5 and 10 days for trisomy 13 and between 4 and 14.5 days for trisomy 18 (e.g.<sup>36–38</sup>). Only five to ten percent of the patients survive beyond the first year of life<sup>36</sup> and only one percent of the children with trisomy 18 survive until their tenth birthday<sup>37</sup>.

Pregnancies diagnosed with Turner's syndrome also carry a very high risk of intrauterine death<sup>53</sup>. It is estimated that 99% of the cases end in a natural miscarriage with a peak around gestational week 13<sup>51</sup>. For live-birth cases, the majority (75%) of infants have a mosaic karyotype<sup>52</sup>. Considering the high percentage of intrauterine fetal deaths, it is thought that a certain degree of mosaicism is necessary for survival in early pregnancy<sup>53</sup>. In agreement with this, a high percentage of Turner patients with mosaicism has been found to be misdiagnosed as 45, X0<sup>54</sup>. Therefore, we specifically searched the literature for the life expectancy of Turner's syndrome patients that were confirmed to be non-mosaic, but this information could not be retrieved.

Fetuses diagnosed with triploidy have three sets of chromosomes. Between one and three percent of clinically recognized pregnancies are estimated to be triploid, but most of them are aborted spontaneously in early gestation<sup>28</sup>. Indeed, a study reporting the natural outcome of pregnancies with a diagnosis of triploidy showed that all fetuses died in the early second trimester between 14 and 18 weeks of gestational age<sup>54</sup>. Studies using birth defect registers confirm that long fetal survival until the third trimester is uncommon<sup>35</sup> and only a few cases of live births (3%) have been reported<sup>39</sup>.



Live birth cases mostly die at an early postnatal stage such that a survival of more than 60 days is very rare<sup>40–42</sup>.

Ideally for group 2, cases with trisomy 13, trisomy 18, Turner's syndrome and triploidy should be analyzed separately since the different chromosomal regions involved may have a different effect on FA. Unfortunately, this was not possible here since sample sizes are too small (even though our 20-year dataset contains more than 1000 cases). Grouping these cases based on comparable fetal survival prognosis likely gives reliable estimates of the potential effect on FA. The average level of FA is also reported for these aneuploidies separately, to provide a superficial idea of whether patterns are similar or not, but we refrain from formal statistical testing.

**Statistical analysis.** Because the age (and thus also the size) of the fetuses and infants in our dataset varied widely, we examined relationships between the unsigned unbiased FA and the average size of each trait. As expected, the unsigned unbiased FA was significantly positively correlated with trait size for femur ( $r = 0.22$ ,  $N = 1015$ ,  $P < 0.001$ ), tibia ( $r = 0.33$ ,  $N = 963$ ,  $P < 0.001$ ), fibula ( $r = 0.25$ ,  $N = 533$ ,  $P < 0.01$ ), radius ( $r = 0.24$ ,  $N = 1016$ ,  $P < 0.001$ ), ulna ( $r = 0.21$ ,  $N = 992$ ,  $P < 0.001$ ), digit 2 ( $r = 0.31$ ,  $N = 174$ ,  $P < 0.001$ ), and digit 4 ( $r = 0.25$ ,  $N = 310$ ,  $P < 0.001$ ). Therefore, we applied a size-correction by dividing each unsigned unbiased FA value by the average trait size and multiplied by 100 (see also<sup>22</sup>). The size-corrected unsigned unbiased FA measures were then standardized and averaged across traits (mean FA). Note that mean FA is based on all available traits, which can vary among individuals due to missing values (e.g. digit 2 could only be measured approximately in one fourth of the cases). Individuals for which mean FA was based on solely one trait were excluded. The mean FA was used as dependent variable to test for differences between the studied groups of chromosomal abnormalities in an ANOVA model. We added age (log-transformed) to the model as previous research detected a significant negative relationship between FA and age<sup>22</sup>. We controlled for possible effects of FA in cases with deficient amniotic fluid levels by adding its presence or absence as categorical variable to the model<sup>43</sup>. Also, sex was included as categorical variable. This model was run twice, first adding individuals without congenital anomalies as group 0, and then using individuals with a normal karyogram. Degrees of freedom of the F-test and the standard errors of the parameter estimates were corrected with the Kenward and Roger method<sup>55</sup>. Pair-wise comparisons were carried out with the Tukey-Kramer adjustment. All analyses were conducted in SAS 9.2 (www.sas.com). The final model was obtained after stepwise backward selection of non-significant factors<sup>56</sup>. Descriptive statistics are reported as mean  $\pm$  SE.

**Ethical statement.** Patient data and radiographs were used according to the guidelines of the Medical Ethics Committee of the VU University Medical Center and patient anonymity was strictly maintained. Parental written informed consent was obtained for patients and data were handled in a coded and completely anonymous fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Data, Dutch Federation of Medical Scientific Societies; <http://www.federa.org/codes-conduct>).

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## Acknowledgments

J.B. is a postdoctoral fellow with the Fund for Scientific Research – Flanders (FWO-Vlaanderen). We owe many thanks to Jaap van Veldhuisen and Ron Otsen of the photography division of the Department of Pathology of the VU University Medical Centre in Amsterdam for the high-quality radiographs and their help digitalizing the radiographs.

## Author contributions

F.G. and S.V.D. designed the study. J.A.M.B. and M.B. managed collections of radiographs and medical databases. J.B. and C.M.A.T.B. performed measures on radiographs. F.G., C.M.A.T.B. and J.B. extracted information from medical databases. J.B. and C.M.A.T.B. analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript. J.B. and C.M.A.T.B. revised the paper.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Bots, J. *et al.* Higher limb asymmetry in deceased human fetuses and infants with aneuploidy. *Sci. Rep.* **4**, 3703; DOI:10.1038/srep03703 (2014).



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