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# Correlation of Anti-Müllerian hormone serum concentration measured in proestrus and estrus with the litter size as a fertility marker in bitches

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ABSTRACT

The use of the anti-Müllerian hormone (AMH) serum concentration as a fertility marker has been shown in cows, sheep and mares and has been indicated in one study in female dogs. The aim of this study was to investigate the connection between the AMH serum concentration, taken at two defined time points during the bitch`s heat, and the litter size to investigate whether AMH can be used as a practical measurement for the individual breeding bitch to predict litter size. The study was carried out on 27 healthy female dogs presented for pre-breeding examination, considering all previously known influencing factors on AMH in the bitch such as age, body weight and estrous cycle phase at the time the sample was taken. Due to the AMH increase in early proestrus and its drop around ovulation, AMH was measured in blood samples taken within the first three days of heat (AMH1) and near ovulation (AMH2) with AMH1 being significantly higher than AMH 2 ($p < 0.001$). There was a highly significant negative correlation of body weight and AMH at both sampling times ($p < 0.001$). There were no significant results when the dogs were paired and grouped according to high or low AMH concentrations, but a significant effect of AMH serum concentration on litter size was found in the multifactorial analysis when the dogs were matched according to their body weight (AMH1: $p = 0.022$; AMH2: $p = 0.030$). In conclusion, a significant effect of the AMH concentration and the litter size among female dogs with matching body weight could be found, but a much larger sample collection is needed to evaluate reference intervals for AMH for bitches of different weight to predict the fertility of an individual bitch in the future.

## 1. Introduction

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein secreted by the granulosa cells of the ovarian follicles in females and the Sertoli cells of the testes in males [1]. In recent years AMH determination replaces the measurement of follicle stimulating hormone (FSH) as a reliable endocrine marker for ovarian reserve in human medicine [2]. In veterinary medicine AMH has been described as a fertility marker in cows and mares to select suitable individuals for superovulation programs in cows and donator mares for embryo transfer [3–5]. In dogs AMH can be used to prove cryptorchidism and ovarian remnant syndrome, to diagnose tumorous degeneration of granulosa and Sertoli cells and to determine the castration status of individuals with unknown history [6–13]. In one study the use of AMH as a predictor of litter size by taking AMH samples at a variable point during estrus has been evaluated [14]. The authors showed that within one breed size, bitches with higher AMH values had larger litter than bitches with lower AMH concentrations. For each 1 ng/ml increase in AMH the litter size increased by 0.3 pups. Another study examined, among other things, AMH during pregnancy in relation to litter size in 13 bitches. The pregnant bitches were divided into three groups. G1A (1-2 puppies), G1B (3-4 puppies) and G1C (5-11 puppies). AMH was measured on the first day of mating and on day 12 of gestation. There was no difference in the AMH levels between the pregnant and non-pregnant bitches. On day 12 of pregnancy, group G1B had higher AMH levels than G1A. However, group G1C, the group with the largest number of puppies, again showed lower AMH values than G1B.

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Body weight was not taken into account in the groups and was examined separately in relation to AMH [15].

Furthermore, changes of the AMH concentration over the estrus cycle in intact bitches have been described [16,17]. The highest AMH concentrations were reached in the last three weeks before the onset of heat until six days before ovulation, which represents the phase of growing pre-antral and small antral follicles, with high inter-individual and intra-individual variation [16,17]. Further Walter et al. (2019) [17] showed a significant decrease over the last three days before ovulation. With this knowledge the time of serum sample collection for AMH measurement should be chosen carefully to get comparable results. The aim of this study was to collect blood serum samples for AMH measurement at certain points in heat and to correlate them with the litter size.

## 2. Materials and methods

### 2.1. Animals

For the study 27 healthy, sexually mature bitches, presented to our clinic for pre-breeding examination, were examined. They belonged to 17 different breeds which were Newfoundland (n = 4), Boxer (n = 3), Rhodesian Ridgeback (n = 3), Kooikerhondje (n = 2), Chinese Crested (n = 2). German Pinscher (n = 2), French Bulldog (n = 1), Jack Russel Terrier (n = 1), Parson Russel Terrier (n = 1), Bouvier des Ardennes (n = 1), Border Collie (n = 1), Bullterrier (n = 1), Dobermann (n = 1), Flat Coated Retriever (n = 1), Dalmatian (n = 1), Chesapeake Bay Retriever (n = 1) and PON (n = 1). They were at the age of 2 years (n = 4), 3 years (n = 3), 4 years (n = 7), 5 years (n = 5), 6 years (n = 6) and 7 years (n = 2). Because of the knowledge, that bitches with lighter bodyweight have higher AMH concentrations [14,17], the bitches were divided in groups of <15 kg (n = 7), 15-30 kg (n = 10) and > 30 kg (n = 10). All bitches had at least one normal estrous cycle previously, 15 bitches had one or more litters so far and there werent any abnormalities in their current estrous cycle. None of the bitches had a systemic disease. All bitches, except of two, were mated with stud dogs that all had sired one or more litters within the last year. Natural mating took place in every case except of one, which got artificial insemination.

### 2.2. Blood sample collection

All blood samples were collected during routine pre-breeding examinations.

The first examination was performed between day one and three of heat. Further examinations were undertaken every two to three days starting on day seven or eight until ovulation took place. All bitches were examined two to six times during proestrus and estrus. The pre-breeding examinations included the documentation of behavior, the inspection of the vulva, vaginoscopy, vaginal cytology and the determination of serum progesterone concentration. The first occurrence of serosanguinous vaginal discharge represented the beginning of proestrus. Serum progesterone concentration was the main parameter to determine the first day of estrus as well as ovulation. Blood samples were centrifuged by 600 G for ten minutes, the supernatant was taken and divided into two aliquots. One sample was immediately used for progesterone determination, the other one was stored by -20°C for AMH measurement later on. All owners provided signed consent for the collection of data for the purpose of treatment and care of animals, as well as for research and agreed, that the collected serum samples for progesterone measurement may be used additionally for AMH measurement.

### 2.3. Hormone analysis

Serum progesterone concentrations were measured with an enzyme linked fluorescence assay (miniVidas; Inc. Biomerieux, Marcy-lÉtoile, France), which has been validated for the bitch [18]. The start of estrus was defined as the rise of progesterone above 2 ng/ml. Ovulation took place at serum progesterone concentrations between 8 and 10 ng/ml and with progesterone concentrations higher than 10 ng/ml ovulation was confirmed.

Because of highest AMH concentrations at early proestrus and the significant decrease over the last three days before ovulation [17], AMH was measured in the serum samples taken on day one to three (AMH1) and the ones taken nearest to ovulation (AMH2), which means one day before, the day of or one day after ovulation. Out of this results the decrease of AMH (AMHd) from proestrus to ovulation was calculated. The main focus was on the AMH1 values, as these represent the early phase of folliculogenesis and thus the development of potentially fertilizable eggs [16].

Measurement of AMH was performed at a commercial laboratory (Laboklin GmbH & Co. kg, Bad Kissingen, Germany). Serum concentrations of AMH were determined using a chemiluminescence immunoassay on a cobas E602 analyser (Roche Diagnostics Deutschland GmbH) using murine anti-AMH-antibodies. The test was validated for dogs [17]. Minimum detection limit of the AMH test was 0.01 ng/ml and maximum detection limit was 23 ng/ml. This test was also used to examine AMH concentrations throughout the estrus cycle of the bitch.

### 2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS 29.01 software. First, the data were tested for normality using the Shapiro-Wilk test. No data set showed a significant deviation from a normal distribution. The mean and standard deviation were calculated for descriptive statistical summary of the data. A paired sample t-test was used to compare AMH serum concentrations at the two time points. In a primary unifactorial analysis, the correlation coefficient Pearson r was calculated to detect possible associations between litter size AMH1 and AMH2, as well as body weight and age of the bitches. Since body weight had a significant relationship with both litter size and AMH concentrations at both time points, bitches with approximately the same body weight were paired and subjected to a multifactorial matched pairs analysis for those parameters with unifactorial significant relationships. This was done using the generalized linear model (GLM) in the variation for repeated measurements (GEE). Litter size was set as the dependent variable, and body weight and AMH concentration at both time points were the predictor variables. In addition, a possible interaction between body weight and AMH1 as well as AMH2 was tested. A significance level of $p = 0.05$ was chosen.

## 3. Results

The mean AMH1 serum concentration of the 27 bitches was 1.36 ± 0.63 ng/ml, which was significantly higher (26.9 %) than the AMH2 serum concentration of 0.99 ± 0.50 ng/ml ($p < 0.001$)(Table 1). Three bitches did not become pregnant, but in none of the cases was there an abnormally low AMH serum concentration. These animals were not included in the following unifactorial correlation analysis of litter size with the other measured parameters.

Ovulation occurred in all bitches examined, which was confirmed using progesterone.

Correlation analysis revealed highly significant negative correlations between body weight (BW) and AMH concentrations at both sampling times (BW-AMH1: $r = -0.68$, $p < 0.001$; BW-AMH2: $r = -0.60$, $p < 0.001$) as shown in Fig. 1. No significant relationship could be found between age and AMH concentrations (age-AMH1: $r = -0.291$, $p = 0.141$; age-AMH2: $r = -0.227$, $p = 0.255$).

The strongest influence on litter size is body weight ($r = 0.543$, $p = 0.006$) as shown in Fig. 2, whereas no significant influence is found for age on litter size ($r = 0.205$, $p = 0.337$). At the same time, a higher AMH serum concentration seems to be associated with a decrease in litter size

**Table 1**
Overview of all taken samples and calculation of the AMH decrease from proestrus to ovulation listed in ascending order of weight.

| **Breed** | Weight in kg | Age in years | **AMH1 ng/ml early proestrus** | **AMH2 ng/ml ovulation** | **AMHdecrease ng/ml AMH2 – AMH1** | **Litter size** |
|---|---|---|---|---|---|---|
| Jack Russel Terrier | 5 | 5 | 1.23 | 1.03 | -0.2 | 3 |
| Parson Jack Russel | 7 | 6 | 2.32 | 2.06 | -0.26 | 4 |
| Kooikerhondje | 7 | 2 | 2.66 | 2.35 | -0.31 | 4 |
| Chinese Crested | 8 | 2 | 2.52 | 1.41 | -1.11 | 5 |
| French Bulldog | 10 | 4 | 1.74 | 1.02 | -0.72 | 3 |
| Chinese Crested | 12 | 5 | 0.99 | 1.02 | 0.03 | 4 |
| Kooikerhondje | 15 | 6 | 1.89 | 1.24 | -0.65 | 6 |
| German Pinscher | 17 | 4 | 1.66 | 1.4 | -0.26 | 7 |
| Border Collie | 17 | 5 | 1.15 | 0.81 | -0.35 | 5 |
| PON | 19 | 3 | 0.94 | 0.42 | -0.52 | 6 |
| German Pinscher | 20 | 3 | 1.33 | 1.19 | -0.14 | 9 |
| Boxer | 25 | 2 | 2.1 | 1.49 | -0.61 | 10 |
| Bullterrier | 25 | 5 | 1.06 | 0.7 | -0.36 | - |
| Dalmatian | 26 | 6 | 1.7 | 0.76 | -0.94 | 10 |
| Bouvier des Ardennes | 27 | 4 | 1.83 | 0.98 | -0.85 | 6 |
| Boxer | 27 | 4 | 1.86 | 1.26 | -0.6 | 6 |
| Dobermann | 29 | 4 | 1.11 | 0.75 | -0.36 | 7 |
| Boxer | 31 | 6 | 0.71 | 0.79 | 0.08 | 7 |
| Flatcoated Retriever | 32 | 6 | 1.88 | 1.6 | -0.28 | 10 |
| Rhodesian Ridgeback | 36 | 6 | 0.75 | 0.36 | -0.39 | 11 |
| Chesapeake Bay Retriever | 36 | 5 | 1.26 | 0.87 | -0.39 | 11 |
| Rhodesian Ridgeback | 39 | 7 | 0.41 | 0.27 | -0.14 | 14 |
| Rhodesian Ridgeback | 40 | 4 | 1.06 | 0.61 | -0.45 | - |
| Newfoundland | 53 | 2 | 0.65 | 0.6 | -0.05 | 10 |
| Newfoundland | 58 | 3 | 0.56 | 0.53 | -0.03 | 6 |
| Newfoundland | 59 | 7 | 0.76 | 0.8 | -0.04 | 6 |
| Newfoundland | 59 | 4 | 0.55 | 0.48 | -0.07 | - |
| | | | mean AMH1 ng/ml | mean AMH2 ng/ml | mean AMH decrease | |
| | | | 1.36 | 0.99 | | |
| | | | | | 26,9 % | |

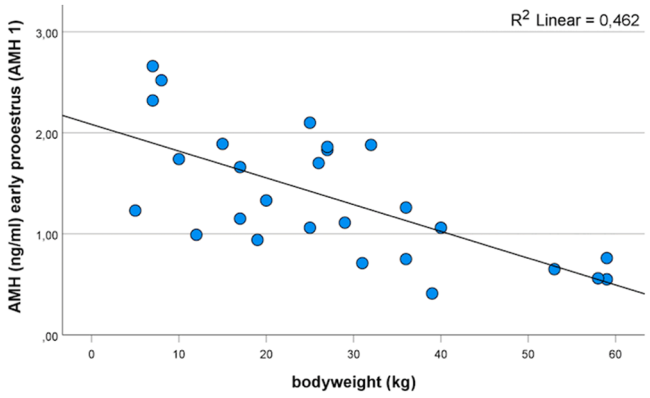


**Fig. 1.** Scatter plot showing the relationship between body weight and the serum concentration of AMH.

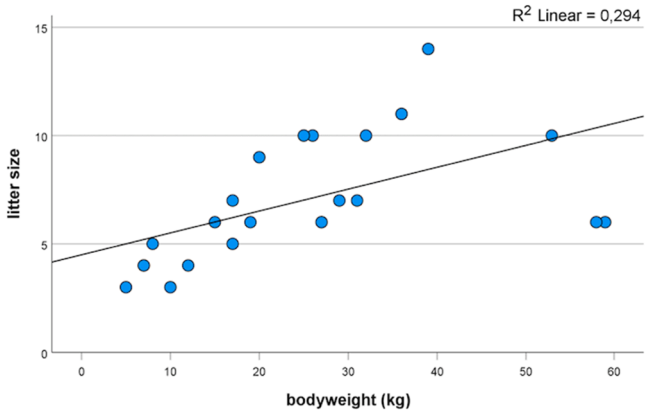


**Fig. 2.** Scatter plot showing the relationship between body weight and litter size.

shown in Fig. 3, although this effect is not clearly significant (litter size-AMH1: r = -0.356, p = 0.088; litter size-AMH2: r = -0.405, p = 0.050).

The matched pairs were divided into two groups according to the different AMH1 serum concentrations within the pairs, group 1 with lower AMH1 serum concentrations and group 2 with higher AMH1 serum concentrations. The mean body weight of these two groups was almost identical, with a difference of 0.16 kg The mean AMH1 concentration in group 1 was 1.08 ± 0.55 ng/ml and was significantly higher in group 2 at 1.65 ± 0.62 ng/ml (paired t-test $p < 0.001$). The mean AMH2 serum concentration was also significantly lower in group 1 at 0.77 ± 0.45 ng/ml than in group 2 at 1.22 ± 0.47 ng/ml ($p < 0.001$).

The multifactorial analysis (GEE) using the matched pairs of bitches with similar body weights showed no significant effect of body weight on litter size (p = 0.355), confirming that the goal of creating matched pairs to significantly reduce the effect of body weight on litter size was achieved. Thus, a significant effect of AMH serum concentration on litter size was found (AMH1: p = 0.022; AMH2: p = 0.030; Fig. 4). The parallel test for an interaction between body weight and AMH serum concentration showed that there was a highly significant interaction at both time points (interaction BW-AMH1: p = 0.001; interaction BW-AMH2: p = 0.016). This means that the independent variable "AMH serum concentration" has a highly significant different effect on the outcome "litter size" depending on the values of the independent variable "body weight".

## 4. Discussion

The aim of this study was to investigate whether AMH can be used as a practical relevant predictor for litter size as a marker for fertility in the individual bitch when the blood sample collection takes place on precisely defined times in heat of the bitches. Therefore, the serum AMH concentrations were determined in early proestrus (AMH1) and around

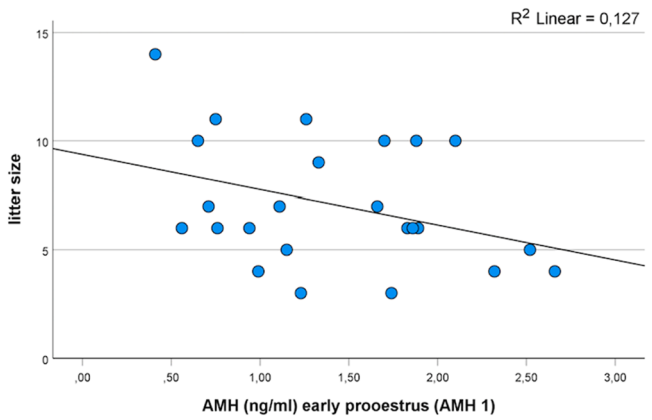


**Fig. 3.** Scatter plot showing the relationship between AMH serum concentration and litter size.

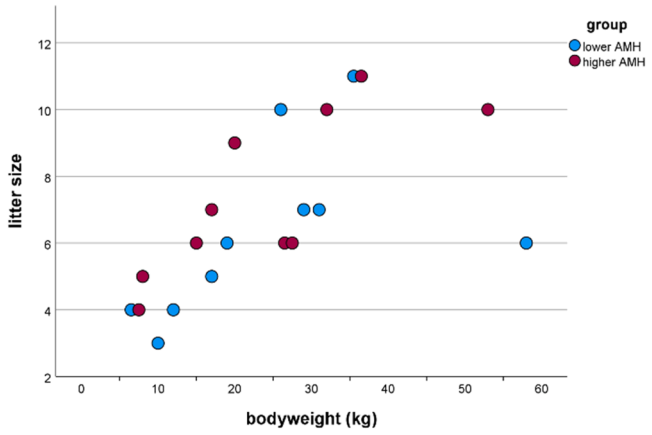


**Fig. 4.** Scatter plot showing the larger litter size in the bitches with the higher AMH serum concentration from the different matched pairs.

ovulation (AMH2) and set into correlation with the litter size. In addition, the effect of weight and age on AMH serum concentration was examined. The average AMH1-value in early proestrus was 1.36 ± 0.63 ng/ml. Near ovulation the average AMH2-value was 0.99 ± 0.50 ng/ml. This matches the results of Walter et al. (2019) [17] using the same test procedure as this study. A narrow reference interval (0.21 – 0.99 ng/ml) was determined with another human ELISA validated for dogs in the study of Nagashima et al. (2016) [16]. In contrast, Hollinshead et al. (2017) [14] used a canine specific ELISA and got a higher and more broad reference interval (11.6 ± 5.6 ng/ml).

This study showed a highly significant negative correlation between body weight and AMH at both sampling times. This coincides with three other studies [14,15,17] but exceptions were seen when some bitches with a high body weight reached as high AMH1-values as bitches with a lower body weight. An explanation for lower AMH values in female dogs with a higher weight could be that although there are a larger number of follicles on the ovary that are ready for ovulation, the AMH they secrete is distributed over a disproportionately larger blood volume. We have to assume that a dog twice as large does not have an ovary twice as large or twice as many follicles. As body mass increases, the AMH value decreases due to the larger blood volume.

The strongest influence on the litter size was the body weight in this study. With an increase in body weight the litter size also increased. This is also shown in the study of Borge et al. (2011) [19]. However, above a body weight of 50 kg the litter size became relatively smaller again in the present study. This is similar to the study by Hollinshead et al. (2017) [14], where bitches above 40 kg had poorer fertility. When examining the AMH progression from early proestrus to ovulation, an average AMH drop of 26.9 % was shown. But the AMH drop was sometimes very small and two bitches even showed a slight increase. This average AMH decrease to ovulation is also described in the other studies by Walter et al. (2019) and Nagashima et al. (2016), where AMH concentration declined sharply back to baseline values beginning 4 days prior to the LH surge.

A general influence of comparatively high AMH values on the size of the litter could not be confirmed in the present study. And even the fact that three bitches failed to get pregnant could not be associated with excessively low AMH1 or AMH2 values in comparison to the bitches which got pregnant. However, if comparing the AMH values of bitches with matching body weight there is a significant effect on the litter size. The correlation of AMH and litter size has only been examined in the study by Hollinshead et al. (2017) [14] so far. The authors have described an increase of the litter size by 0.3 puppies for every 1 ng/ml AMH. It should be noted that the study by Hollinshead et al. (2017) [14] was carried out with a canine ELISA test, which leads to a much wider reference interval of AMH (2.9-21.1 ng/ml). Also, the number of 155 bitches sampled compared to this study may lead to a higher accuracy. Besides, the sampling times in the study of Hollinshead et al. (2017) [14] were variable over estrus, whereas the present study is based on precise sampling times, which, if one considers the course of AMH in proestrus and estrus, is not insignificant for the level of AMH concentration. Thus, at the beginning of estrus, you can still get quite high AMH values, whereas you can detect low AMH values just before ovulation [16]. Therefore, sampling at random times in estrus may have an effect on the correlation with litter size. Even during pregnancy, AMH does not seem to be able to give a reliable indication of litter size. In the study carried out for this purpose, the bitches with medium-sized litters had higher AMH values on day 12 of pregnancy than the bitches with small litters, but the bitches with the largest litters had lower AMH values. Body weight, which has a decisive influence on the level of AMH values, was not taken into account but was assessed separately [15].

Looking at current studies from human medicine which deal with AMH and fertility, other possible reasons for the lack of a correlation between AMH and fertility in the bitch are possible. In human medicine, AMH is used as an indirect marker to estimate the ovarian functional reserve and to predict the onset of menopause [20]. It should be mentioned here that AMH only allows statements to be made about the functional ovarian reserve (FOR) and not about the actual ovarian reserve. The FOR describes the pool of follicles that have a size of 2 to 5 mm and can therefore be stimulated by FSH [21]. Actual follicular reserve is described as the number of dormant primordial follicles [21]. As an indirect marker of the functional ovarian reserve, AMH is subject to influencing factors which not always affect the actual ovarian reserve, but can possibly be transferred to veterinary medicine. For example, in women AMH values 18 % higher were measured in the summer, which is why a connection with the vitamin D level is suspected [22]. A study of women between the ages of 18 and 40 also found out that women with comparatively low AMH values were just as likely to become pregnant as women with high AMH values [23]. Another study found large differences in fertility of women despite similar AMH levels [24]. In human medicine, AMH is used more to find out if there are follicles that are potentially ready to rupture, but does not allow any statement to be made about the quality of the oocyte, so it is not necessarily suitable as a predictor for the probability of pregnancy [20,25]. Since AMH does not provide any information about the quality of the oocytes, this is a possible explanation why in the present study bitches with high AMH values do not automatically have proportionally larger litters. However, after comparison of bitches with similar body weight there was a significant correlation and therefore in the bitch there might be an informative value of the AMH concentration with regard to fertility. But this finding needs to be confirmed in future studies with a larger sampling size.

The average decrease in AMH concentration from the fourth year of

life described by Hollinshead et al. (2017) [14] could not be seen in this study. The results of this study are more consistent with the results of the study of Turna et al. (2015) [7], in which no age-related AMH decline was found.

A general and gynecological examination of all bitches, as well as history taking, should have minimized other factors affecting fertility. However, it cannot be ruled out that circumstances such as being kept in a pack, other stress factors or a drop in progesterone during pregnancy led to smaller litter sizes. This would have required a standardized ultrasound examination, which was also not mentioned in the study of Hollinshead et al. (2027) [14]. The sperm quality of the male dogs should also be taken into account when interpreting this study. Using breeding dogs that have successfully bred within the last year should reduce the likelihood of smaller litters due to poor sperm quality. A standardized examination of the sperm would have allowed more precise results.

## 5. Conclusion

Body weight has the strongest influence on litter size and there is a highly significant negative correlation between the AMH value and the body weight. This leads to a significant effect of the AMH concentration and the litter size among female dogs with matching body weight.

However, a much larger sample collection is needed to evaluate reference intervals for AMH within the different weight groups to predict the fertility of an individual bitch in the future.

## CRediT authorship contribution statement

**Theresa Hornberger:** Writing – original draft, Methodology, Data curation. **Sven Reese:** Formal analysis. **Klaus Perbandt:** Formal analysis. **Andrea Meyer-Lindenberg:** Supervision. **Beate Walter:** Supervision, Methodology.

## Declaration of competing interest

The authors have no conflicts of interest and there are no financial conflicts of interest.

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