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Human ovarian reserve from conception to the menopause.

Authors

Wallace WHB* (1) and Kelsey TW (2)

Addresses

1 Division of Child Life and Health, Department of Reproductive and Developmental Sciences, University of Edinburgh, Scotland, UK.

2 School of Computer Science, University of St. Andrews, Scotland, UK.

*To whom all correspondence should be addressed at:

Dr W. Hamish B. Wallace, Consultant Paediatric Oncologist, Royal Hospital for Sick Children, Sciennes Road, Edinburgh EH9 1LF.

E-mail: hamish.wallace@nhs.net

Summary paragraph

Current understanding is that the human ovary contains a fixed number of several million non-growing follicles (NGF), established by five months of gestational age, that declines with increasing age to the menopause when approximately 1,000 NGF remain at an average age of 50-51 years[1,2]. With approximately 450 ovulatory monthly cycles in the normal human reproductive lifespan, this progressive decline in NGF numbers is attributed to follicle death by apoptosis. Individual histological studies[3-10] have quantified NGF numbers over limited age ranges. However, no model describing the rate of establishment and decline of the NGF population from conception to menopause has been previously reported. Here we describe the best fitting model of the age-related NGF population in the human ovary from conception to menopause. Our model matches the log-adjusted NGF population to a five-parameter asymmetric double Gaussian cumulative (ADC) curve ($r^2 = 0.81$). Furthermore we found that the rate of NGF recruitment into growing follicles for all women increases from birth until approximately age 14 years (coinciding with puberty) then decreases towards the menopause. The explanation for this new finding remains unclear but is likely to involve both paracrine and endocrine factors. We describe and analyse the best fitting model for the establishment and decline of human NGF; our model extends our current understanding of human ovarian reserve.

Text

Our current understanding of mammalian ovarian reserve presumes that the human ovary establishes several million non growing follicles (NGF) at around five months of gestational age which is followed by a decline to the menopause when approximately 1,000 remain at an average age of 50-51 years[1,2]. With approximately 450 ovulatory monthly cycles in the normal human reproductive lifespan, this progressive decline in NGF numbers is attributed to follicle death by apoptosis. Some studies have suggested that the instantaneous rate of temporal change increases around the age of 37 years, when approximately 25,000 follicles remain, followed by exhaustion of the NGF pool and menopause 12-14 years later[6,7]. Several recent studies have challenged this long held understanding of mammalian reproductive biology by reporting the presence of mitotically-active germ stem cells in juvenile and adult mouse ovaries[11,12,13]. While the presence of germ stem cells within the mammalian ovary that are capable of neo-oogenesis remains controversial[14,15], no-one has previously attempted to unify the establishment and decline of NGF in the human ovary from conception to menopause in one biologically plausible mathematical model.

Several studies have reported the number of NGF's at different ages in humans[3-10] and constructed mathematical models of NGF decline[1,2,8]. Most recently a study

by Hansen et al. (2008) described a model that predicts no sudden change in the rate of decline, but rather a constantly increasing rate[8]. In this study we have combined the eight known published studies (Table 1) that have counted the number of NGF in human ovaries from conception through birth to menopause. We have then fitted asymmetric peak models to the data (Supplementary Table 1) and selected the highest ranked to produce a new model to describe the establishment and decline of human ovarian reserve (Figure 1a). The highest ranked model ($r^2 = 0.81$) is a 5-parameter asymmetric double-Gaussian cumulative (ADC) curve (Supplementary Figure 1). The model is asymmetric, since rapid establishment is followed by a long period of decline; it is double-Gaussian cumulative since it is the product of two Gauss-error functions. The highest ranked model for the establishment and decline in NGF population from conception to menopause was found to be:

$\log_{10}(NGF) = (a/4)(1 + erf((x-b + c/2)/(d\sqrt{2})))(1 - erf((x-b - c/2)/(e\sqrt{2})))$, with coefficients a = 5.56 (95% CI 5.38-5.74), b = 25.6 (95% CI 24.9-26.4), c = 52.7 (95% CI 51.1-54.2), d = 0.074 (95% CI 0.062-0.085), and e = 24.5 (95% CI 20.4-28.6). For this model, n = 325, $r^2 = 0.81$; fit standard error = 0.46; F-value = 364 (Supplementary Figures 1 and 2; Supplementary Table 2). Our model demonstrates that 81% of the variation in individual NGF populations is due to age alone. Interestingly, if we confine our analysis to the histological data from conception to

age 25 years we discover that the ADC model remains the best fit ($r^2 = 0.95$) and that 95% of the variation in NGF numbers is due to age alone (Figure 1b). To guard against model selection bias, and to test the robustness of the model with respect to the data, we have shown that the ADC model provides the best fit of any asymmetric peak function to our dataset ($p < 0.01$, Supplementary Table 3).

We hypothesise that the age at menopause for an individual woman is determined by the maximal number of NGF's that are established at 18-22 weeks post-conception, followed by a decline according to our ADC model until near exhaustion at the menopause (Supplementary Figure 3). If menopause is defined as a population of less than one thousand, the model predicts age of menopause as 49.6 (95% CI 47.9 – 51.2) years, with a 95% prediction interval of 38.7 – 60.0 years (Figure 2). Our model has a maximum NGF population for the average age at menopause of 300,000 (95% CI 225,000 – 390,000), occurring at 18-22 weeks post-conception, with a 95% prediction interval (PI) of 35,000 - 2,534,000 NGF's. Figure 2 gives values for NGF populations at illustrative ages, together with the corresponding 95% prediction intervals. Women with an average age of menopause (50-51 years) will have around 295,000 NGF present at birth, with women destined to have an earlier menopause having around 35,000 NGF and late

menopause women having over 2.5 million NGF at birth. These numbers are per ovary, so total ovarian reserve is approximately double these populations.

Since the publications by Johnson et al. (2004,5)[11,12] there has been lively scientific debate around the widely held concept that a non-renewing oocyte reserve is laid down in the ovaries at birth, and that neo-oogenesis does not occur in adult life[14]. Johnson and Tilly have argued that their experiments in the adult female mouse have demonstrated conclusively that neo-oogenesis continues in adulthood. They have proposed that the source of postnatal oocyte production is from germline stem cells in the bone marrow which are transported in the peripheral circulation as germline progenitor cells to arrive in the adult ovary[12]. The recent report showing isolation and culture of germ line stem cells from adult mouse ovaries[13], which restored fertility after injection into infertile mice, provides further evidence to support the presence of germ line stem cells in mammalian ovaries. Our further analysis of the available histological data demonstrates that any mathematical model that permits an increase in NGF population after the peak at 18-22 weeks has a markedly inferior fit compared to the best-fitting asymmetric peak functions (Supplementary Figure 4). While the emerging evidence strongly supports the existence of germ stem cells within adult

mouse ovaries[15], our model provides no supporting evidence of neo-oogenesis in normal human physiological ageing.

We describe the percentage of the NGF population remaining for a given age for all women whose ovarian reserve is established and declines in line with our model (Figure 3). It is interesting to note that by the age of 30 years the percentage NGF population is already 12% of the initial reserve and only 3% of the reserve remains at 40 years of age. A recent study has shown that most women underestimate the extent to which age affects fertility and pregnancy outcomes[16].

In order to better understand which factors control recruitment of NGF towards selection, growth and ovulation we have investigated the monthly rate of decline of NGF from birth to menopause. To our knowledge, the number of NGF recruited each month has never been reliably estimated. By solving our model we can show (Figure 4a) that the maximum recruitment of 880 NGF's per month occurs at 14 years 2 months for the average age at menopause. While the maximum rate of recruitment varies hugely, from around 100 NGF per month (Figure 4b) to over 7,500 NGF per month (Figure 4c) for early or late menopause respectively, the rate of NGF recruitment increases to a plateau at just over 14 years and then decreases in all women irrespective of how many NGF were established by birth. This peak

at 14 years is highly unlikely to be coincidental. In western society the average age of menarche is around 13 years[17], with early breast development appearing around age 11 years. We have found that the onset of oestrogenisation and ovulation heralds a slowing in the rate of NGF recruitment. Our findings strongly suggest that both endocrine and paracrine factors may be important in the slowing and subsequent decline in the rate of NGF recruitment. An important candidate is anti-Mullerian hormone (AMH), a member of the transforming growth factor-beta (TGF-beta) superfamily of growth factors[18]. They are produced by ovarian granulosa cells and oocytes in a developmental, stage-related manner and function as intra-ovarian regulators of folliculogenesis. There is good evidence that AMH from granulosa cells of pre-antral or antral follicles exerts a negative inhibitory influence on the primordial to primary follicle transition[19]. Furthermore AMH has been proposed as an indirect marker of ovarian reserve in post-pubertal women[19]. Until the onset of puberty (characterised by the switching on of the hypothalamic-pituitary axis and the pulsatile secretion of the gonadotophins FSH and LH) follicular maturation rarely progresses beyond the pre-antral stage. The presence of the pulsatile secretion of FSH and LH at puberty promotes follicular maturation to the antral stage and beyond. There is however incomplete data on AMH levels in pre-pubertal girls in the literature: AMH is undetectable before birth[20] and is detectable at low levels in infants[21]. The explanation for our finding that the rate

of NGF recruitment increases until the onset of puberty, levels off at around 14 years of age, and then declines to the menopause remains unclear. It is interesting to speculate that AMH levels which are undetectable at birth may rise at puberty with the establishment of regular ovulatory cycles and be responsible for the slowing of the rate of NGF recruitment that occurs at puberty.

Can a more complete understanding of the establishment and decline of the non-renewing pool of NGF help us to assess ovarian reserve for the individual woman? Several candidate markers for the assessment of ovarian reserve in the individual woman have been suggested including FSH, Inhibin B, AMH, and antral follicle counts and ovarian volume by transvaginal ultrasound[22]. We recently showed a striking correlation between ovarian volume and NGF population using an earlier model[23]. However the measurement of ovarian volume by transvaginal ultrasound is imprecise, particularly at the lower end of the range[24]. It is likely that a better understanding of NGF establishment and decline will improve our ability to assess ovarian reserve for the individual woman. One immediate application of our model is to better understand the effect of chemotherapy and radiotherapy on the human ovary. Using a model based on less complete histological data, we estimated the radiosensitivity of the human oocyte[25] and were subsequently able to estimate the effective sterilising dose of radiotherapy at a given age for the individual

woman[26]. Knowledge of the dose of radiotherapy and age at which it is delivered provides an important opportunity for accurate counselling of women receiving cancer treatment and will help us to predict which women are at high risk of premature menopause and who may therefore benefit from ovarian cryopreservation[27].

In summary, we have identified the first biologically plausible model of ovarian reserve from conception to menopause that fits the combined histological evidence. This model allows us to estimate the number of NGF present in the ovary at any given age, suggests that 81% of the variance in NGF populations is due to age alone, and shows for the first time that the rate of NGF recruitment increases from birth to age 14 years then declines with age until menopause.

Methods Summary

Data was collected from eight separate quantitative histological studies (Table 1). Each of these studies used a variation on the technique developed by Block[3]. Ovaries are sectioned and some of the sections stained and photographed. A mean follicular volume is calculated from a sample image and used throughout all subsequent calculations. The photographs are analysed by hand, with the number of NGF's appearing in the photograph being counted. By assuming an even distribution throughout the ovary, the population of the samples is integrated into an estimated population for the entire ovary. The studies differ in the stain used, the number of samples chosen, the method of counting, and the mathematical formula used to obtain the estimated population from the sample populations. No standard error has been calculated for such studies, as the exact number of NGF's in a specific ovary has not been calculated. We combined these data into a single dataset (n=325) (Supplementary Table 4) and enforced a zero population at conception.

We fitted 20 asymmetric peak models to the data set, using TableCurve-2D (Systat Software Inc., San Jose, California, USA), and ranked by r^2 correlation coefficient (Supplementary Table 3). An asymmetric double-Gaussian cumulative (ADC) model was ranked highest (Supplementary Tables 1, 2, 4, 5 & 6; Supplementary Figures 1 & 2). To avoid selection bias, we randomly removed 50 datapoints 61

times and re-fitted the models, with the ADC model being, on average, the best fitting model (double-sided t-test for the difference of means, $p < 0.01$)(Supplementary Table 3). To further avoid selection bias from our initial choice of models, we fitted all 266 models supplied by TableCurve, again ranking by r^2. The ADC model was again the highest ranked (Supplementary Table 7).

The highest ranked model was used as the basis for further calculations. Under the modelling assumption that, in general, a high (respectively low) established population results in a late (resp. early) menopause (Supplementary Figure 3), we calculated the percentage of NGF pool at given ages (Supplementary Tables 5 & 6), and the absolute monthly loss of germ cells from birth until age 55 (Figure 4).

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Legends to Figures

Figure 1a.

The best model for the establishment of the NGF population after conception, and the subsequent decline until menopause is described by $\log_{10}(NGF) = (a/4)(1 + \text{erf}((x-b + c/2)/(d\sqrt{2})))(1 - \text{erf}((x-b - c/2)/(e\sqrt{2})))$, with parameters a = 5.56 (95% CI 5.38-5.74), b = 25.6 (95% CI 24.9-26.4), c = 52.7 (95% CI 51.1-54.2), d = 0.074 (95% CI 0.062-0.085), and e = 24.5 (95% CI 20.4-28.6).

Our model has correlation coefficient $r^2 = 0.81$, fit standard error = 0.46 and F-value = 364. This figure shows the dataset (n= 325), the model, the 95% prediction limits of the model, and the 95% confidence interval for the model. The horizontal axis denotes age in months up to birth at age zero, and age in years from birth to 51 years.

Figure 1b.

The best model for the establishment of the NGF population after conception, and the subsequent decline until 25 years of age is described by $\log_{10}(NGF) = (a/4)(1 + \text{erf}((x-b + c/2)/(d\sqrt{2})))(1 - \text{erf}((x-b - c/2)/(e\sqrt{2})))$, with parameters a = 5.79 (95% CI 5.03-6.55), b = 28.0 (95% CI 15.8-40.2), c = 57.4 (95% CI 33.1-81.8), d = 0.074 (95% CI 0.067-0.081), and e = 34.3 (95% CI -4.2-72.8).

This model has correlation coefficient $r^2 = 0.95$, fit standard error = 0.29 and F-value = 585. This figure shows the dataset (n= 126), the model, the 95% prediction limits of the model, and the 95% confidence interval for the model. The horizontal axis denotes age in months up to birth at age zero, and age in years from birth to 25 years.

Figure 2.

This figure gives illustrative examples of NGF populations predicted by our model. At ages 20 weeks, birth, 13 years, 25 years and 35 years the average NGF population is given, together with the respective 95% prediction intervals. The predicted average age at menopause (49.6 years) is also shown, together with the 95% prediction interval.

Figure 3.

The curve describes the percentage of ovarian reserve remaining at ages from birth to 55 years, based on the Wallace-Kelsey model. 100% is taken to be the maximum ovarian reserve, occurring at 18-22 weeks post-conception. The percentages apply to all women whose ovarian reserve declines in line with our model (i.e. late and early menopause are associated with high and low peak NGF populations, respectively).

Figure 4

Each sub-figure describes the absolute number of NGFs recruited per month, for ages from birth to 55 years, based on population decline predicted by the Wallace-Kelsey model. Figure 4 (a) – red curve – denotes the average age at menopause case ; maximum recruitment of 880 follicles per month occurs at 14 years 2 months. Figure 4 (b) – green curve – denotes recruitment for individuals whose decline is in line with the 95% lower prediction limit for the model; maximum recruitment of 104 follicles per month occurs at 14 years 2 months. Figure 4 (c) – yellow curve – denote recruitment in line with the upper 95% prediction limit; maximum recruitment of 7,520 follicles per month occurs at 14 years 2 months.

Supplementary Figure 1.

The figure produced by TableCurve for the highest ranked model fitted to the combined dataset. The first three parameters, (a, b and c) define the maximum amplitude, centre and width respectively. The remaining parameters (d and e) define the scale of population establishment and decline.

Supplementary Figure 2.

The first equation is the generic form of an asymmetric double-Gaussian cumulative curve related to log adjusted data. The curve is asymmetric as it is the product of two Gaussian cumulative functions having a shared peak, but having different scale factors for growth and decline. Amplitude parameter **a** controls the maximum height of the curve above the x-axis; location parameters **b** and **c** control the height of the curve either side of the maximum. Scale parameters **d** and **e** control the width of the growth and decline sections of the curve. The second equation is the model obtained by replacing the parameters with computed values that maximise the r^2 correlation coefficient for the 325 data points. The x variable is now age, and the y variable is the log-adjusted NGF population. This is the Wallace-Kelsey model from conception to menopause.

Supplementary Figure 3.

This table describes the hypothesis that individual age at menopause is determined by the peak NGF population established at around 20 weeks post-conception. The central curve is the ADC model described in Figures 1a and 2. Above and below are the hypothetical curves for an ovary having log-adjusted peak population varying from the average case by one half, one, one and a half, and two standard deviations. Under this hypothesis, a variation by, for example, one standard

deviation in the initial peak population results in a one standard deviation from the average age at menopause.

Supplementary Figure 4.

The TableCurve output for the highest ranked model that permits more than one population peak. Compared to the ADC model, this has lower correlation coefficient, higher fit standard error, and lower F-statistic. All other TableCurve models that allow multiple peaks have an inferior fit to the data.

End Notes

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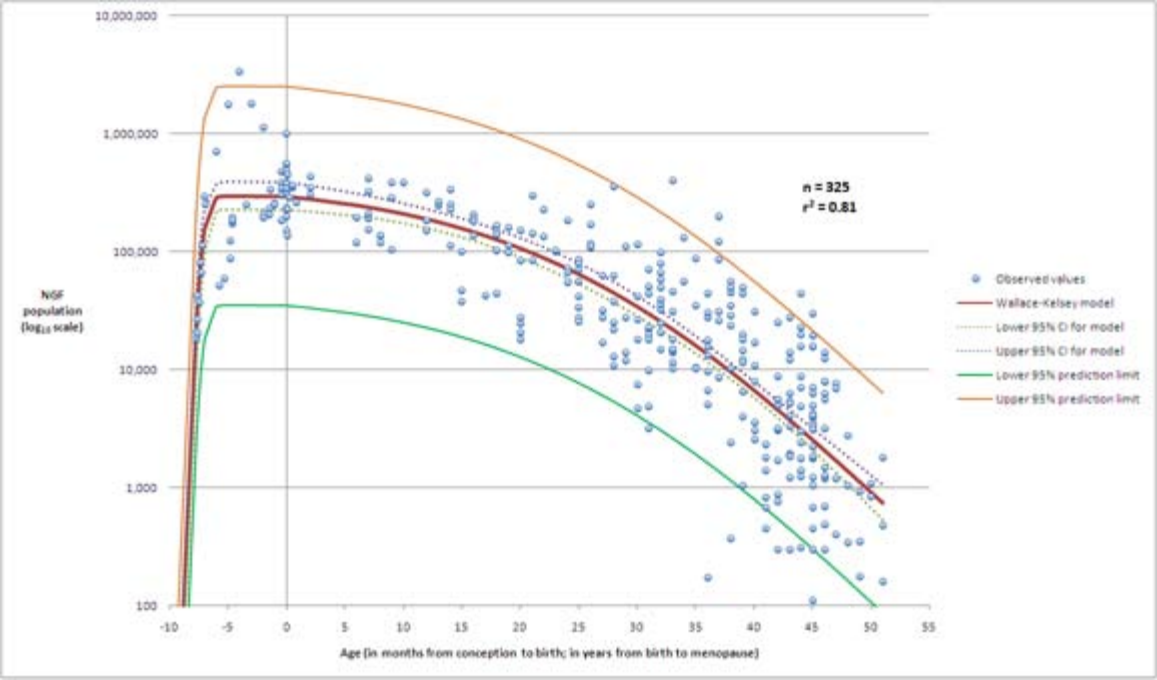
Author contributions

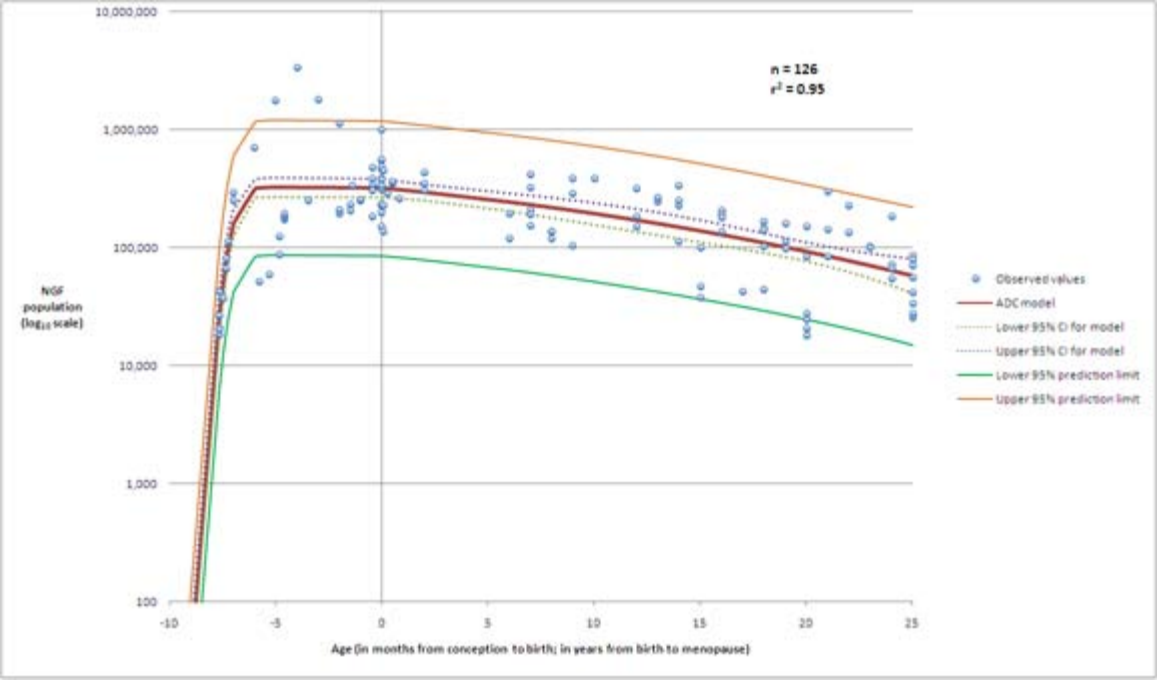
WHBW and TWK jointly conceived the study, analysed the data and wrote the paper.

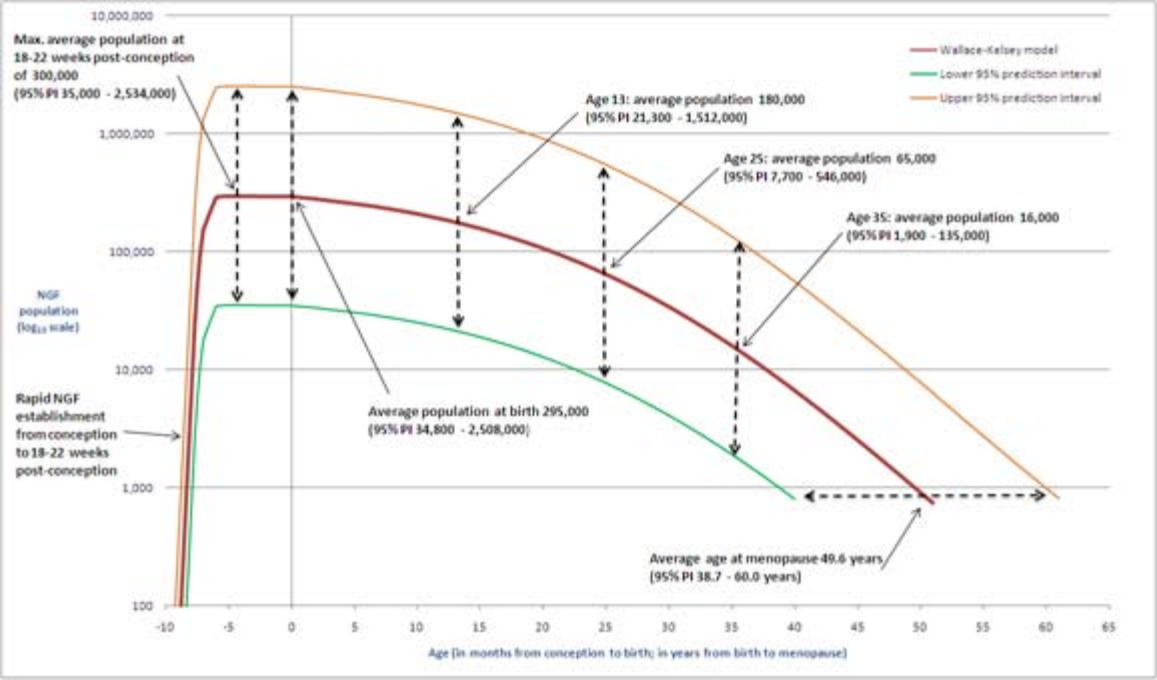
Table One.

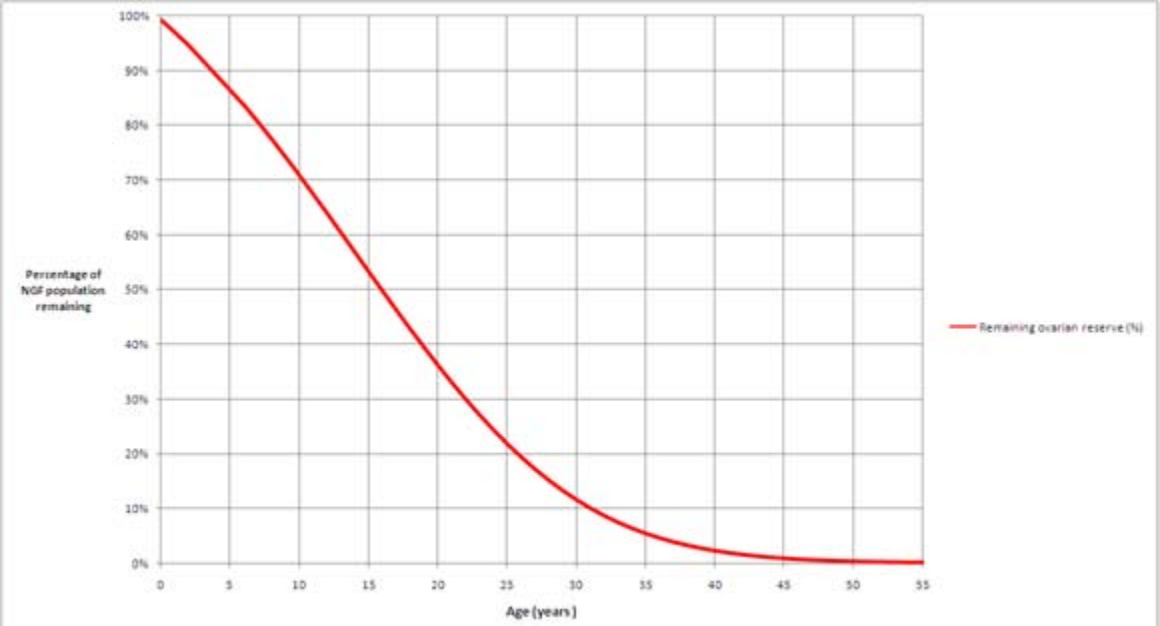
Legend: Details of the eight quantitative histological studies used to form the combined dataset.

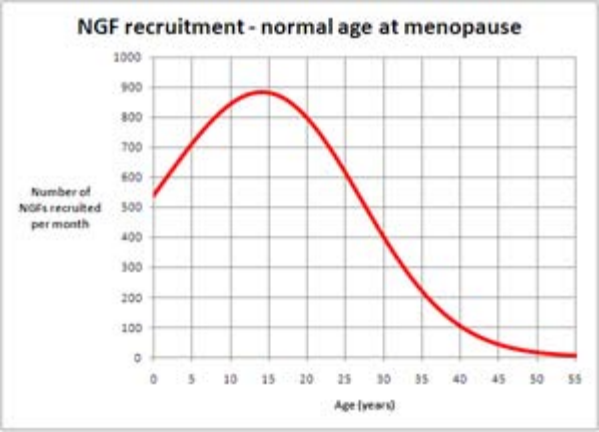
Study			Statistics			
Number	First Author	Year	Ovaries	Min. Age	Max. Age	Median Age
1	Bendsen	2006	11	-0.6	-0.6	-0.6
2	Baker	1963	11	-0.6	7.0	-0.2
3	Forabasco	2007	15	-0.5	0.5	-0.3
4	Block	1953	19	-0.2	0.0	0.0
5	Hansen	2008	122	0.1	51.0	38.0
6	Block	1952	86	6.0	44.0	28.0
7	Gougeon	1987	52	25.0	46.0	39.5
8	Richardson	1987	9	45.0	51.0	46.0
	Overall		325	-0.6	51.0	32.0

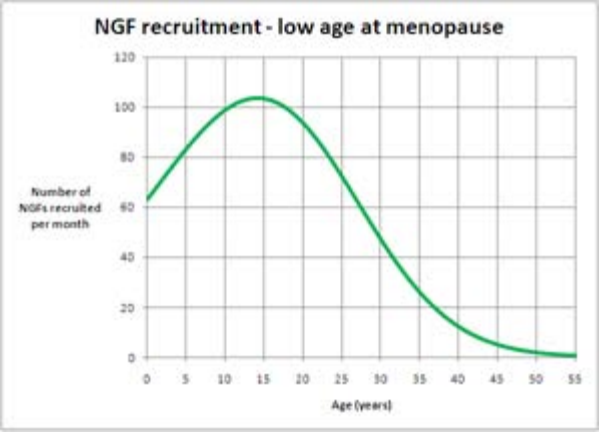

10,000,000
1,000,000
100,000
10,000
1,000
100
NGF population (log_{10} scale)
n = 325
r^2 = 0.81
-10
-5
0
5
10
15
20
25
30
35
40
45
50
55
Age (in months from conception to birth; in years from birth to menopause)
Observed values
Wallace-Kelsey model
Lower 95% CI for model
Upper 95% CI for model
Lower 95% prediction limit
Upper 95% prediction limit

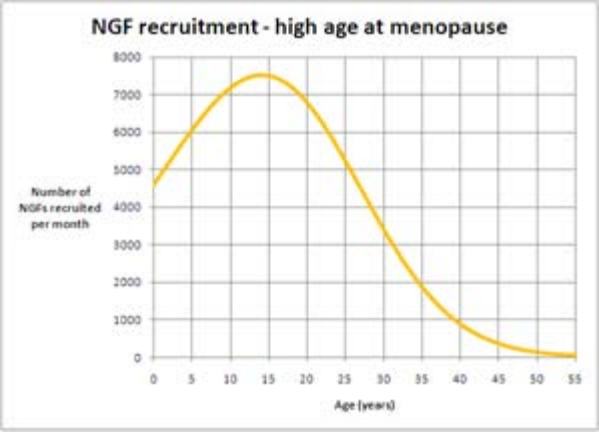

n = 126
r² = 0.95
10,000,000
1,000,000
100,000
10,000
1,000
100
NGF population (log10 scale)
-10
-5
0
5
10
15
20
25
Age (in months from conception to birth; in years from birth to menopause)
Observed values
ADC model
Lower 95% CI for model
Upper 95% CI for model
Lower 95% prediction limit
Upper 95% prediction limit

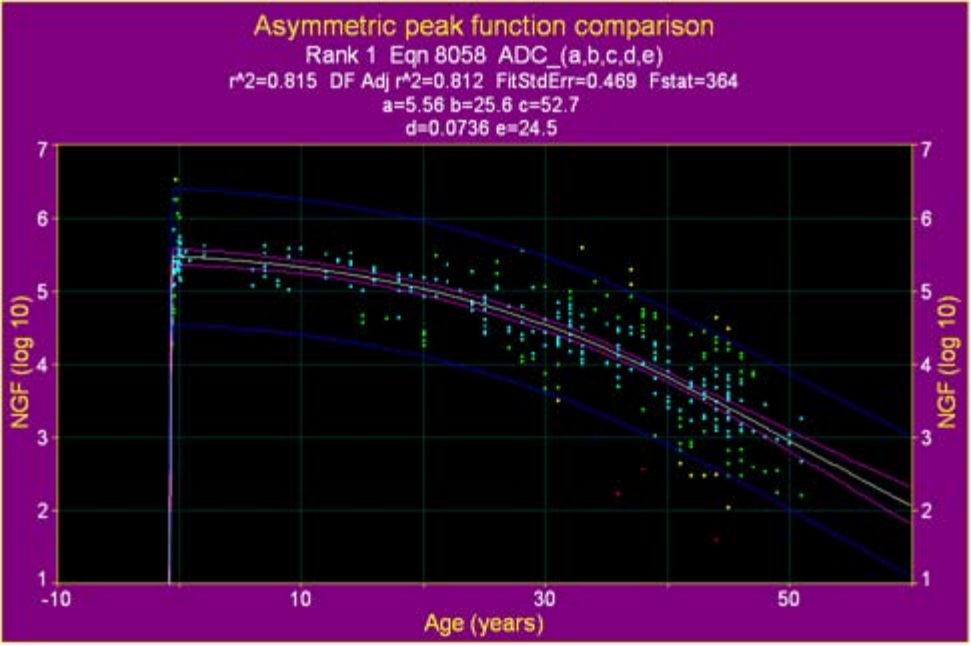

Max. average population at 18-22 weeks post-conception of 300,000 (95% PI 35,000 - 2,534,000)
Age 13: average population 180,000 (95% PI 21,300 - 1,512,000)
Age 25: average population 65,000 (95% PI 7,700 - 546,000)
Age 35: average population 16,000 (95% PI 1,900 - 135,000)
Average population at birth 295,000 (95% PI 34,800 - 2,508,000)
Rapid NGF establishment from conception to 18-22 weeks post-conception
Average age at menopause 49.6 years (95% PI 38.7 - 60.0 years)
Wallace-Kelsey model
Lower 95% prediction interval
Upper 95% prediction interval
NGF population (log10 scale)
10,000,000
1,000,000
100,000
10,000
1,000
100
-10
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Age (in months from conception to birth; in years from birth to menopause)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of NGF population remaining
0
5
10
15
20
25
30
35
40
45
50
55
Age (years)
Remaining ovarian reserve (%)


NGF recruitment - normal age at menopause
Number of NGFs recruited per month
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
55
Age (years)

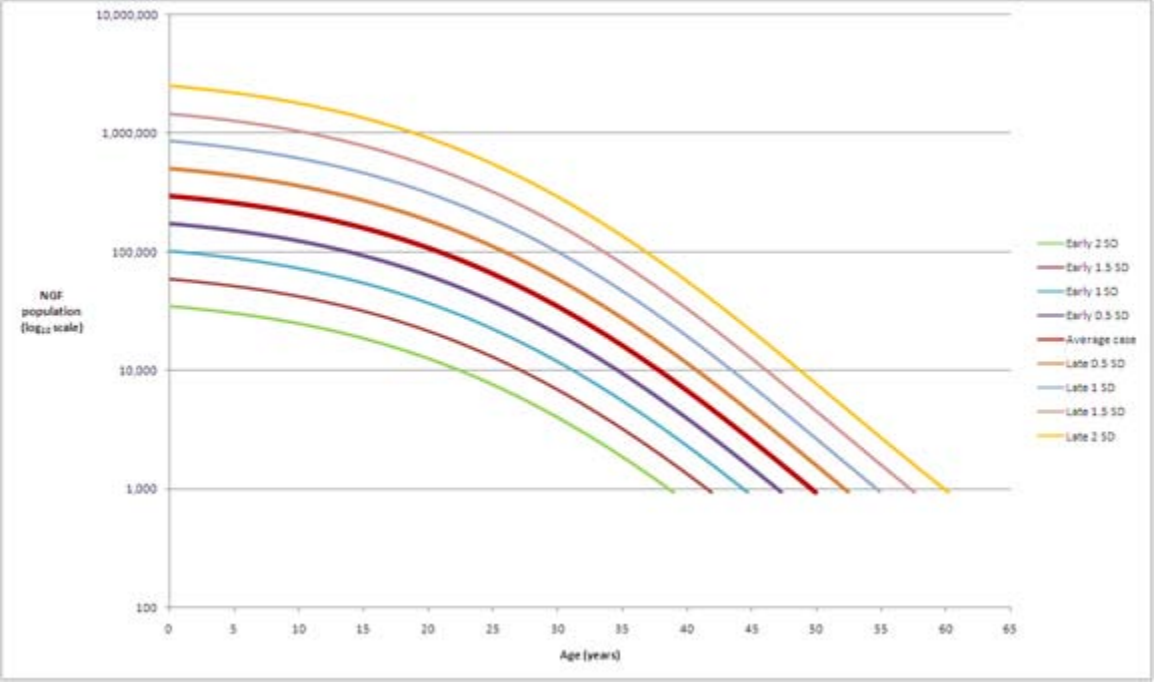

NGF recruitment - low age at menopause
Number of NGFs recruited per month
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
55
Age (years)

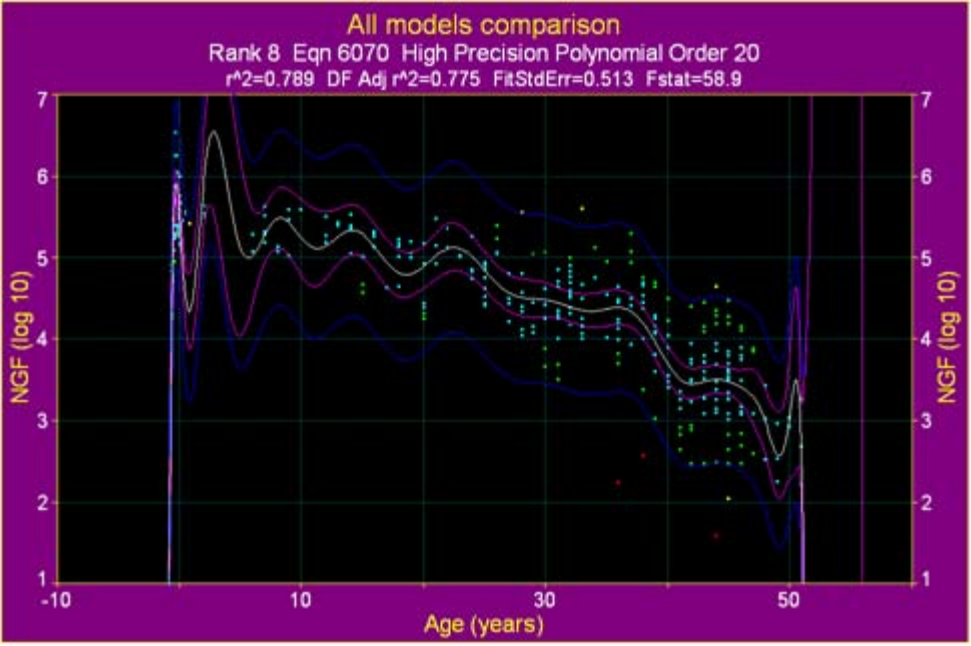

NGF recruitment - high age at menopause
Number of NGFs recruited per month
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
35
40
45
50
55
Age (years)


Asymmetric peak function comparison
Rank 1 Eqn 8058 ADC_(a,b,c,d,e)
r^2=0.815 DF Adj r^2=0.812 FitStdErr=0.469 Fstat=364
a=5.56 b=25.6 c=52.7
d=0.0736 e=24.5
NGF (log 10)
NGF (log 10)
Age (years)
7
6
5
4
3
2
1
-10
10
30
50

$$log_{10}(y) = \frac{a}{4}\left[1 + \text{Erf}\left(\frac{x + b + \frac{c}{2}}{d\sqrt{2}}\right)\right]\left[1 - \text{Erf}\left(\frac{x + b - \frac{c}{2}}{e\sqrt{2}}\right)\right]$$

$$log_{10}(NGF) = \frac{5.56}{4}\left[1 + \text{Erf}\left(\frac{age + 25.6 + \frac{52.7}{2}}{0.074\sqrt{2}}\right)\right]\left[1 - \text{Erf}\left(\frac{age + 25.6 - \frac{52.7}{2}}{24.5\sqrt{2}}\right)\right]$$


10,000,000
1,000,000
100,000
10,000
1,000
100
NGF population (log_{10} scale)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Age (years)
Early 2 SD
Early 1.5 SD
Early 1 SD
Early 0.5 SD
Average case
Late 0.5 SD
Late 1 SD
Late 1.5 SD
Late 2 SD


All models comparison
Rank 8 Eqn 6070 High Precision Polynomial Order 20
r^2=0.789 DF Adj r^2=0.775 FitStdErr=0.513 Fstat=58.9
NGF (log 10)
NGF (log 10)
7
6
5
4
3
2
1
-10
10
30
50
Age (years)