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Evaluation of Serum Anti-Müllerian Hormone (AMH) Values for 28,016 Bulgarian Women: Prognostic Statistical Model of Age Specific AMH Declining

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Abstract

The present study aims to establish a relationship between serum AMH levels and age in a large group of women living in Bulgaria, as well as to establish reference age-specific AMH levels in women that would serve as an initial estimate of ovarian age. A total of 28,016 women on the territory of the Republic of Bulgaria were tested for serum AMH levels with a median age of 37.0 years (interquartile range 32.0 to 41.0). For women aged 20 - 29 years, the Bulgarian population has relatively high median levels of AMH, similar to women of Asian origin. For women aged 30 - 34 years, our results are comparable to those of women living in Western Europe. For women aged 35 - 39 years, our results are comparable to those of women living in the territory of India and Kenya. For women aged 40 - 44 years, our results were lower than those for women from the Western European and Chinese populations, close to the Indian and higher than Korean and Kenya populations, respectively. Our results for women of Bulgarian origin are also comparable to US Latina women at age 30, 35 and 40 ages. On the base on constructed a statistical model to predicting the decline in AMH levels at different ages, we found non-linear structure of AMH decline for the low AMH < 1 group, while for the other two AMH groups (1 ≤ AMH ≤ 3.5 and AMH > 3.5) the dependence of the decline of AMH on age was confirmed as linear. In conclusion, we evaluated the serum level of AMH in Bulgarian women and established age-specific AMH percentile reference values based on a large representative

sample. We have developed a prognostic statistical model that can facilitate the application of AMH in clinical practice and the prediction of reproductive capacity and population health.

Keywords

Anti-Müllerian Hormone, Women Age, Ovarian Response, Ethnicity, Prognostic Statistical Model

1. Introduction

In recent decades, demographic and socio-economic changes have affected the role of women in society. Women have been assuming an increasingly active role in society, and the birth of a first child often is postponed for years. However, as a woman ages, the function of the ovary decreases, along with the ovarian reserve. This typically leads to a more difficult pregnancy and more generally, to an increase in the necessity for infertility treatment through assisted reproductive techniques (ART).

As a matter of fact, IVF technology is the main method of treating infertility. Approximately 10% of women have an unsatisfactory response to gonadotropin stimulation [1] [2]. The main reason is the reduced ovarian reserve in women, which in most cases is related to a woman's age. In routine practice, various methods are used to estimate the reserve, the main ones being ultrasound determination of the number of antral follicles and ovarian volume, as well as basal levels of FSH and Inhibin B in the blood, determined during menstruation [3] [4] [5]. Another marker is the Anti-Müllerian hormone (AMH), which has emerged as a major biomarker related to ovarian reserve.

The anti-Müllerian hormone is a dimeric glycoprotein produced by granulosa cells of the antral follicles in the ovaries, and AMH participates in the formation of the dominant follicle during folliculogenesis [6]. AMH is produced in the ovary by preantral or small antral ovarian follicles [7]. Serum AMH levels are relatively constant and show little variability between and within the menstrual cycle but are affected by a woman's weight [8]. Studies have found that women who are overweight or have a higher BMI have lower levels of AMH than women of the same age with a BMI that is in the normal range [9] [10]. Taking contraceptives can also affect AMH levels [11].

The level of serum AMH is proportional to the number of antral follicles and therefore it is most suitable for determining ovarian reserve [12]. For this reason, AMH is a convenient marker to reflect the biological age of the ovary and the response to controlled ovarian stimulation [13].

A single AMH determination is a prognostic marker for the oocyte quantity associated with ovarian stimulation during an in vitro procedure. Serum levels of AMH have a prognostic value for the ovarian response to stimulation, and on this basis, several authors divide the patient cases into several groups [14] [15].

Very low responders—AMH < 0.5 ng/mL, with a prognosis of unsatisfactory ovarian response during IVF and the formation of no more than three follicles [16] [17] [18], which in turn reduces the chance of pregnancy;

Low responders—AMH < 1.0 ng/mL, which is associated with a limited number of oocytes during stimulation and is not related to the woman's age [19] [20].

Normal responders—AMH = 1.1 - 3.4 ng/mL, with the formation of multiple follicles and aspiration between 8 - 15 oocytes per stimulation [21] [22].

High responders—AMH > 3.4 ng/mL with formation of multiple follicles and aspiration of 15 or more oocytes. The clinical features of polycystic ovaries (PCOS) and an increased risk of OHSS during stimulation are common in high responders. In these cases, specific stimulation protocols are applied to reduce this risk [23] [24].

Factors such as race and ethnicity also have an impact on AMH levels. Studies have found differences in AMH levels in different North American groups, respectively among white, black, and Hispanic women [25] [26]. These demographic variables indicate a need to establish reference ranges for AMH for individual populations in different geographic areas. Data on AMH in the Balkan region are scarce, including for the Bulgarian population. Bulgarians are a mixed multinational population, with 85% identifying them with the Bulgarian ethnicity and the remaining 15% with another ethnicity, including Turkish, Jewish, Armenian, and Gypsy/Roma ethnic groups [27].

The present study aims to establish a relationship between serum AMH levels and age in a large group of women living in Bulgaria, as well as to establish reference age-specific AMH levels in women that would serve as an initial estimate of ovarian age. Based on this analysis, a mathematical model can be created to predict the rate of reduction of AMH levels at different ages. This could help to improve family planning and family building [28], and potentially improve discussions between patients and practitioners regarding informed expectations and the consideration of fertility treatment options.

2. Materials and Methods

A retrospective study was conducted between January 2018 and April 2023. A total of 28,016 women were included in this retrospective study, with samples collected and analyzed in Cibalab and Synevo Bulgaria laboratories. These are two of the three largest laboratories in Bulgaria that provide analysis of samples for serum AMH, according to the requirements of local clinics and hospitals in Bulgaria. The two laboratories have blood collection points all over the country and have a different structure, with Cibalab being one centralized laboratory with 38 blood collection points, and Synevo having 4 laboratories located in different parts of the country with 44 blood collection points. Data on serum AMH levels were anonymized before analysis. The study participants were women from an Eastern European population living in Bulgaria.

2.1. Investigational Sites

AMH measurements were performed in 28,016 (Cibalab—23,528 and Synevo—4488) randomly selected women aged 20 to 50 years. Each woman was represented only once in a set of 28,016 AMH samples. In the analysis of the results, the median, the average, and interquartile range (IQR) parameters were determined at one-year intervals from 20 to 50 years of age and also for age groups, as follows: 20 - 24, 25 - 29, 30 - 34, 35 - 39, 40 - 44, and 45 - 50 years. Results were analyzed in subgroups with AMH values < 1.0 ng/mL, between 1.0 and 3.5 ng/mL, and concentrations > 3.5 ng/mL at one-year intervals in subsets of women aged 20 to 50 years old.

AMH analysis in Cibalab was performed using the Ansh Labs (Webster, TX, USA) manual assays, including the ultra-sensitive AMH enzyme-linked immunosorbent assay (ELISA); measuring range 0.1 - 16.0 ng/ml. The intra-assay imprecision (repeatability) was 1.97% for a concentration of 0.35 ng/mL and 4.0% for a concentration of 1.85 ng/mL. The between run imprecision (intermediate precision) were 4.63% and 1.98%, respectively.

Synevo laboratories analyzed serum AMH levels using the Elecsys AMH automated method on a clinically validated platform (Cobas e 402 and e 801, Roche Diagnostics, Germany) with Intra- and inter-assay coefficients of variation with serum controls were approximately 1.2% and 1.7% and 2.2% - 4.4%, respectively and management range 0.01 - 23 ng/mL.

2.2. Statistical Analysis

We estimated summary statistics, including median and several quantiles for each age-year group, as well as for the six age-range groups.

Based on the observed decline with age of the median age-year AMH in different groups, first we modeled the AMH dynamics in the different groups with a generalized additive model with Gaussian kernel. This confirmed the nonlinear characteristics of the AMH decrease for the low AMH < 1 group, while the other two groups ($1 \leq AMH \leq 3.5$ and AMH > 3.5) the dependence of AMH decline with age was confirmed to be linear. Based on these results, we estimated a predictive model for AMH that has two stages, as follows. For each of the three AMH-level groups above, we estimated the parameters of a separate statistical model: for the two groups with higher AMH levels, we estimated separate robust linear models, while for the low AMH < 1 group, we estimated a segmented robust linear model with three segments, which allows for different estimated rate of decline of AMH for three age ranges. The estimated ages at which the slope of rate decline changes were approximately 33 and 42 years. The final predictive model proceeds with two stages, where first the candidate is assigned to the appropriate AMH-level group, followed by prediction using the appropriate robust linear model for that group.

All statistical analyses were performed using the R statistical language.

2.3. Ethics

All study and sample collection sites followed the guidelines of the International

Conference on Harmonization for Good Clinical Practice E6 and conducted the study in accordance with the Declaration of Helsinki (as amended by Tokyo, Venice, Hong Kong, and Edinburgh). Blood sampling and serum testing had clinical purposes, and all patients consented to data analysis for publication. The authors had no direct contact with patients in this study, and patient data was anonymized before the authors had access to it, and no patient identifying information was disclosed. The present study was a retrospective, observational study, therefore it was not deemed necessary to seek ethics committee approval.

3. Results

A total of 28,016 women on the territory of the Republic of Bulgaria were tested for serum AMH levels with a median age of 37.0 years (interquartile range/IQR/ aged 32.0 to 41.0). The number of women was highest in the 35–39 age group (28.9%), followed by 40 - 44 years (27.2%). The smallest group includes women aged 20 - 24 (3.1%). The largest number of women in one-year intervals were at the ages of 38 (6.2%), 39 (6.1%), and 40 (6.1%), and the least is at the age of 20 (0.3%).

The highest values of AMH were found at the age of 21 years with an average of 7.141, a median of 7.4, and IQR (4.48 - 10.08). Single-year-specific median AMH and reference intervals (5^{th}-95^{th}) percentiles are summarized in Table 1.

The results were determined, the median, average parameters, and interquartile range (IQR) of group ages intervals, respectively: 20 - 24, 25 - 29, 30 - 34, 35 - 39, 40 - 44, and 45 - 50 years. Additionally, cases were divided into subgroups according to serum AMH levels: less than 1 ng/ml, between 1.1 and 3.5 ng/ml, and more than 3.5 ng/ml (Table 2). The percentage distributions of AMH in the different subgroups at different ages are summarized in Table 3.

The results show that median AMH values decrease with advancing age.

The results of the samples determining the serum levels of AMH between the two laboratories Cibalab and Synevo Bulgaria were compared (Table 4). In the analysis, no statistically significant difference was found in AMH levels in one-year intervals from 20 to 50 years of age, excepting cases with AMH < 1 ng/ml at ages 31 and 33, and cases with AMH > 3.5 ng/ml at ages 24, 27, 30, 33 and 37 years. We believe that the reason for the presence of a significant difference in the AMH levels of these subgroups of women, the small number of samples provided by Synevo laboratory.

4. Analysis

The average annual decline in mean serum AMH is 0.26 ng/mL/year until age 30 and then declines at a rate of 0.22 ng/ml until age 35. The average annual rate of decline in AMH with ages between 35 and 40 is 0.20 ng/mL, and after 41 years it is already on average 0.1 ng/mL/year.

The results for the rate of AMH reduction in the different subgroups are interesting.

Table 1. Single-year-specific median AMH and reference intervals (5th-95th) percentiles.

Age	Median	Quantiles					
		5.00%	10.00%	25.00%	75.00%	90.00%	95.00%
20	6.04	1.69	2.55	4.14	9.08	13.53	16.09
21	7.4	2.26	2.66	4.48	10.08	13.08	16.26
22	6.31	1.46	1.87	3.32	9.7	15	16.6
23	5.46	1.86	2.29	3.8	8.2	12.29	16
24	5.65	1.24	1.6	3.21	9.37	15.93	17.11
25	5.03	0.97	1.51	2.93	7.92	13.39	16.6
26	5.18	0.66	1.36	2.72	8.13	11.77	15.35
27	4.61	0.55	1.34	2.76	7.46	10.87	14.42
28	4.2	0.78	1.25	2.34	6.95	10.88	13.47
29	3.64	0.67	1.08	2.02	6.5	10.02	13.21
30	3.4	0.44	0.76	1.76	6.02	9.89	12.52
31	3.26	0.31	0.64	1.55	5.66	9.35	11.92
32	2.96	0.32	0.63	1.43	5.31	8.45	11
33	2.55	0.23	0.49	1.1	5.02	8.13	10.91
34	2.3	0.16	0.45	1.03	4.35	6.96	10.03
35	2.03	0.14	0.36	0.94	3.96	6.7	9.44
36	1.85	0.12	0.3	0.84	3.62	6.27	8.89
37	1.54	0.05	0.23	0.62	3.1	5.59	7.5
38	1.3	0.05	0.15	0.53	2.77	4.87	6.58
39	1.02	0.05	0.12	0.42	2.32	4.48	5.78
40	0.98	0.05	0.05	0.37	2.01	3.97	5.51
41	0.74	0.05	0.05	0.27	1.64	3.2	4.33
42	0.67	0.05	0.05	0.23	1.45	2.78	3.87
43	0.57	0.05	0.05	0.17	1.22	2.38	3.25
44	0.37	0.05	0.05	0.05	0.95	1.87	2.47
45	0.34	0.05	0.05	0.05	0.83	1.65	2.67
46	0.24	0.05	0.05	0.05	0.6	1.15	1.69
47	0.15	0.05	0.05	0.05	0.47	1	1.54
48	0.05	0.05	0.05	0.05	0.34	0.74	0.87
49	0.05	0.05	0.05	0.05	0.29	0.66	0.89
50	0.05	0.05	0.05	0.05	0.05	0.26	0.38

Table 2. Subgroups according to serum AMH levels: less than 1 ng/ml, between 1.1 and 3.5 ng/ml, and more than 3.5 ng/ml.

	Age Group					
	20 - 24	25 - 29	30 - 34	35 - 39	40 - 45	45+
All						
N	737	2440	5360	6796	6327	1868
Median	6.07	4.37	2.81	1.52	0.68	0.18
Quartile 25%	3.54	2.38	1.33	0.62	0.22	0.05
Quartile 75%	9.2	7.32	5.23	3.16	1.51	0.58
AMH < 1						
N	23	185	1010	2496	3969	1623
Median	0.41	0.52	0.54	0.43	0.32	0.13
Quartile 25%	0.05	0.2	0.24	0.18	0.05	0.05
Quartile 75%	0.81	0.79	0.76	0.69	0.61	0.38
1 ≤ AMH ≤ 3.5						
N	158	779	2124	2815	1902	219
Median	2.47	2.26	2.1	1.88	1.67	1.46
Quartile 25%	1.87	1.66	1.51	1.39	1.27	1.16
Quartile 75%	3.04	2.88	2.72	2.52	2.26	2
AMH > 3.5						
N	556	1476	2226	1485	456	26
Median	7.5	6.54	5.88	5.34	4.89	4.55
Quartile 25%	5.44	4.81	4.47	4.24	4.01	3.93
Quartile 75%	10.3	9.15	8.38	7.33	6.39	5.68

Table 3. The percentage distribution of AMH in the different subgroups at different ages.

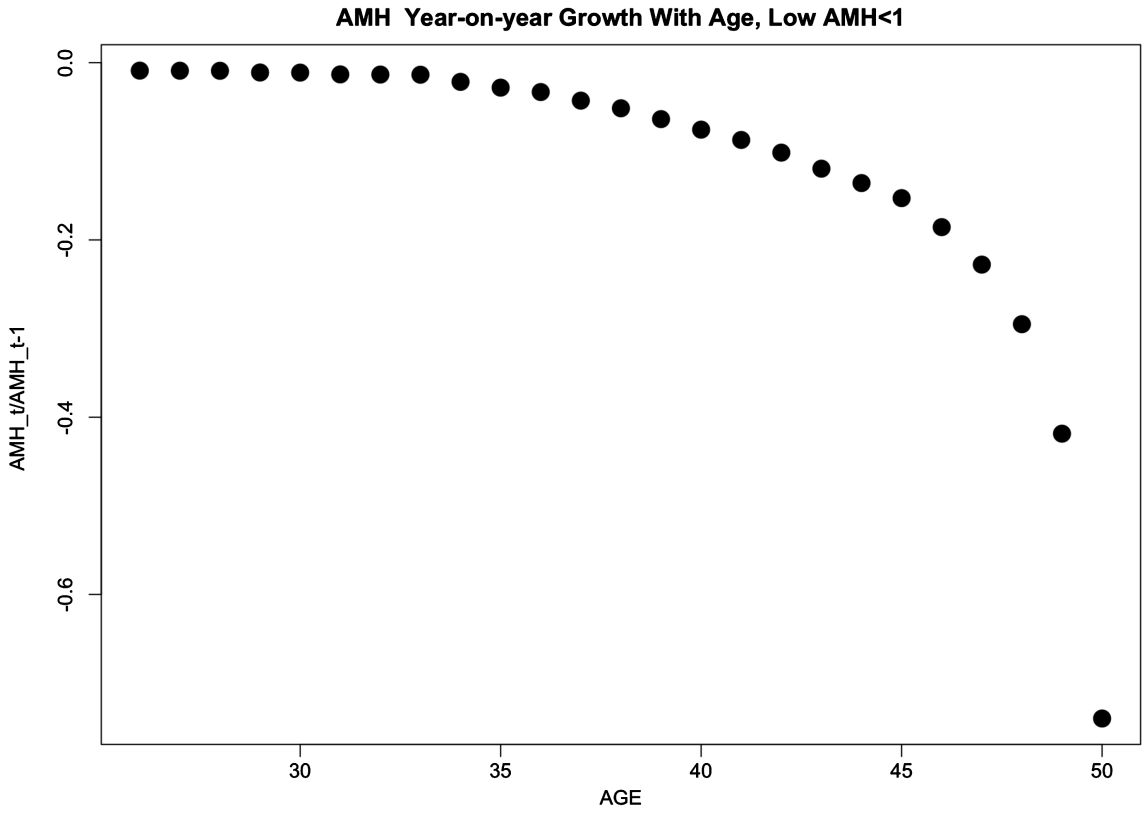
Age Group	20 - 24	25 - 29	30 - 34	35 - 39	40 - 44	45+
N	737	2440	5360	6796	6327	1868
AMH < 1						
N	23	185	1010	2496	3969	1623
N, %	3.1	7.6	18.8	36.7	62.7	86.9
1 < AMH < 3.5						
N	158	779	2124	2815	1902	219
N, %	21.4	31.9	39.6	41.4	30.1	11.7
AMH > 3.5						
N	556	1476	2226	1485	456	26
N, %	75.4	60.5	41.5	21.9	7.2	1.4

Table 4. Comparison between median serum levels of AMH in two different laboratories (Cibalab and Synevo Bulgaria).

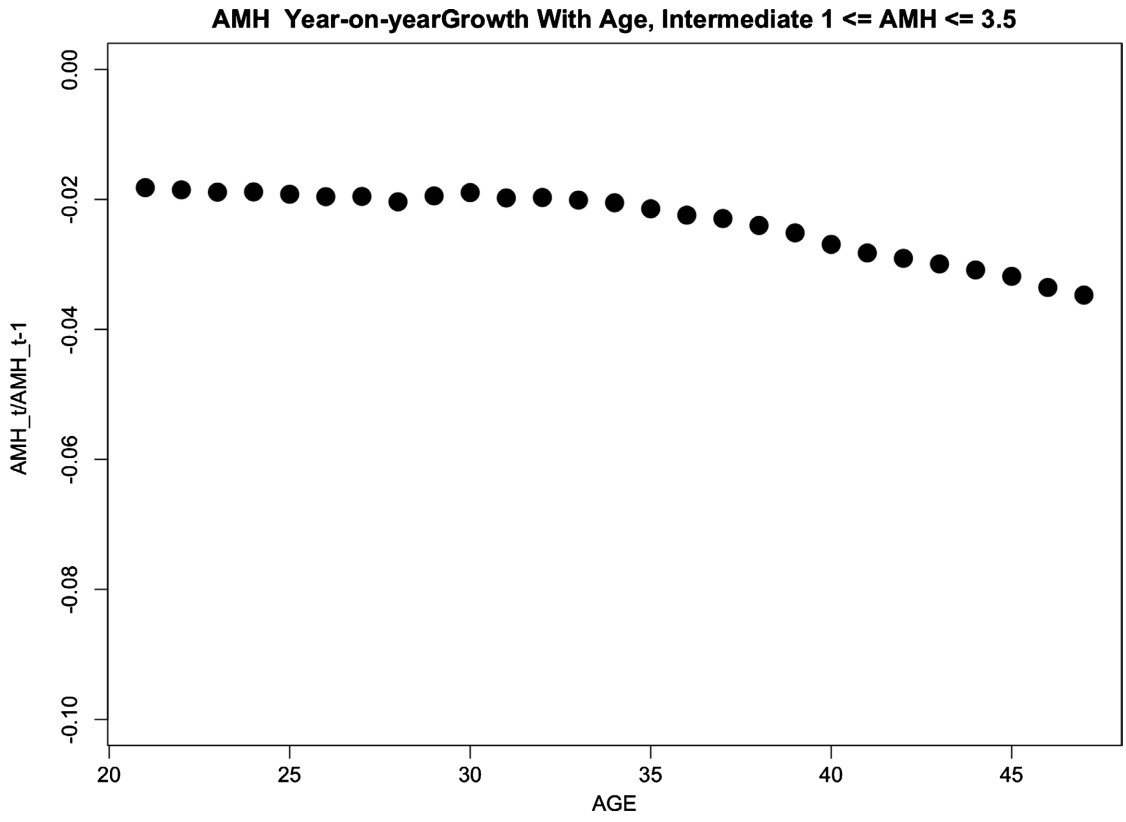
Age	AMH < 1			1 < AMH ≤ 3.5			AMH ≥ 3.5		
	Synevo N cases	Cibalab N cases	P-value	Synevo N cases	Cibalab N cases	P-value	Synevo N cases	Cibalab N cases	P-value
24				18	57	0.065	21	152	0.01
25	10	16	0.064	12	74	0.791	27	197	0.61
26	8	28	0.585	18	91	0.405	22	253	0.12
27	10	37	0.845	39	136	0.141	42	318	0.02
28	14	42	0.154	41	198	0.461	35	342	0.21
29	19	62	0.927	59	280	0.574	51	366	0.28
30	20	123	0.891	65	338	0.316	43	433	0.04
31	41	148	0.029	85	371	0.642	39	467	0.01
32	34	190	0.528	93	454	0.282	47	496	0.04
33	54	257	0.048	101	460	0.557	52	427	0.03
34	80	292	0.226	108	501	0.410	42	403	0.89
35	100	338	0.334	103	564	0.153	38	382	0.2
36	99	375	0.251	112	573	0.179	43	339	0.57
37	136	475	0.062	107	567	0.858	28	285	0.01
38	144	602	0.248	121	588	0.481	30	266	0.24
39	154	706	0.450	109	523	0.747			
40	181	757	0.059	74	533	0.995			
41	214	859	0.475	63	451	0.572			
42	230	896	0.082	72	406	0.110			
43	209	788	0.387	53	324	0.232			
44	158	669	0.564	30	188	0.338			
45	121	504	0.238	15	118	0.603			
46	112	392	0.439	13	55	0.056			
47	74	323	0.593						
48	45	209	0.256						
49	23	111	0.264						
50	20	84	0.668						

In women with AMH < 1 ng/ml, the rate of decline with advancing age changes as follows: until age 33, the decline in AMH is very slow, between ages 33 and 42 there is an acceleration of the process, and after 42 years of age, the rate of decrease of AMH is strongly accelerated (Graphic 1).

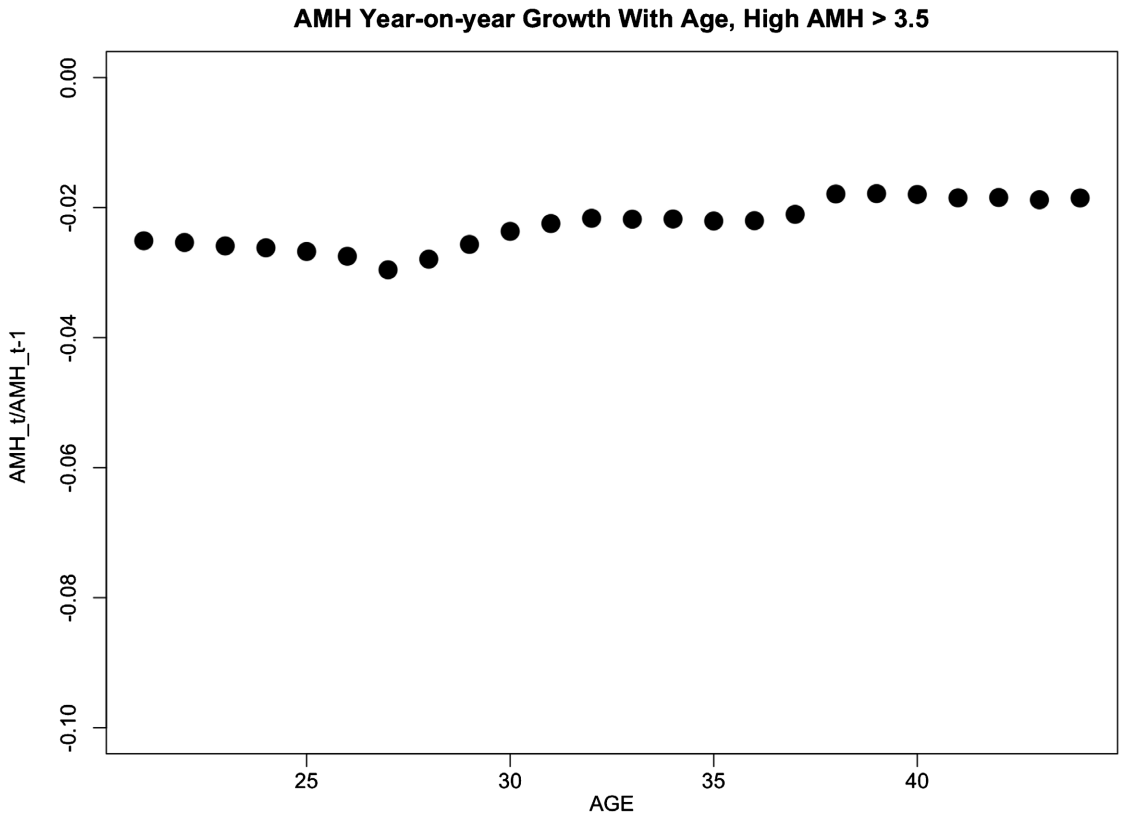
In women with AMH between 1 and 3.5 ng/ml, the rate of decline with age was uniform and constant, as can be seen from the graph of the rate of change of AMH across ages having a linear characteristic (Graphic 2).



Graphic 1. AMH year-on-year growth with age. Low AMH levels less than 1.0 ng/ml.



Graphic 2. AMH year-on-year growth with age. AMH levels between 1.0 ng/ml and 3.5 ng/ml.



Graphic 3. AMH year-on-year growth with age. High AMH levels more than 3.5 ng/ml.

In women with AMH greater than 3.5 ng/ml, the rate of hormone decline also appears uniform and constant, but with a different, faster rate of decline (Graphic 3).

5. Discussion

This study, which is unique to Bulgaria, investigates and analyzes age-specific levels and ranges of serum AMH levels, based on a large statistical sample of 28,016 women of reproductive age. This sample from a population of 7 million people of Bulgarian origin is the largest, percentage-wise, compared to other similar studies. AMH measurements were performed using two methods, in two different laboratory chains. For this reason, we performed a comparative assessment of AMH levels in different ages between the two laboratories, and the results showed no statistically significant difference in AMH levels for the different age groups. In our comparative analysis, however, we compared AMH levels at different ages using two different methodologies. This gives us reason to conclude that the two different methods of assessing AMH levels at different ages are comparable when used in clinical practice. Despite these results of the comparative analysis of the samples from the two laboratories, we did not pool the results in the subsequent analyses. The reason is aiming for maximum robustness of the analysis due to established variations from −25% to +45% at different levels of AMH in the different methods [29] [30] [31].

Thus, for optimal robustness of the estimates, we performed a separate analysis of the samples from only one laboratory, Cibalab, with 23,528 AMH samples. On the one hand, the quantity of samples was still sufficiently large for the analysis, and on the other hand, the samples of serum AMH levels were examined in only one laboratory, using a single methodology.

When analyzing the serum levels of AMH in different age groups of women, we found that the median and interquartile range of AMH in ages 20 - 24 was 6.09 ng/ml (3.54 - 9.20), for 25 - 29 years it was 4.37 ng/ml (2.38 - 7.32), for 30 - 34 years: 2.81 ng/ml (1.33 - 5.23), for 35-39 years: 1.52 ng/ml (0.62 - 3.16), for 40 - 44 years: 0.68 ng/ml (0.22 - 1.51), and for 45-49 years: 0.18 ng/ml (0.05 - 0.58).

Comparing our results with other studies grouped similarly, we find that in women aged 20 - 24 years, the Bulgarian population has relatively high median levels of AMH, similar to women of Asian origin, namely, for women living on the territory of the Republic of Korea. For women aged 25 - 29 years, our results are comparable to those of women living in China. For women aged 30 - 34 years, our results are comparable to those of women living in Western Europe. For women aged 35 - 39 years, our results are comparable to those of women living in the territory of India and Kenya. For women aged 40 - 44 years, our results were lower than those for women from the Western European and Chinese populations, close to the Indian and higher than Korea and Kenya populations, respectively. Our results for women of Bulgarian origin are also comparable to US Latina women at age 30, 35 and 40 ages [32]-[36].

When comparing the percentage ratio of cases of women from the Bulgarian ethnicity with AMH levels ≤ 1.0 ng/ml to the rest, we find that for the age between 20 - 24 years, women with low AMH levels are 3.1%, between 24 - 29: 7.6%, ages 30 - 34: 18.8%, ages 35 - 39: 36.7%, ages 40 - 44: 62.7%, ages 45 - 50: 86.9%.

This percentage of women with low AMH was significantly lower in our study group of women, compared to women of African origin who were treated for infertility [35]. Other studies have also analyzed the percentage of cases with low AMH levels. Authors [32] [37] analyzing the prevalence of cases with reduced ovarian reserve according to the criteria of the American College of Obstetrics and Gynecology (ACOG) and the American Society for Reproductive Medicine (ASRM), found that the relative percentage of women with AMH < 1 ng/mL and FSH > 10 mIU/mL in different age groups are as follows: in the group 20 - 24 years: 3.8%, 25 - 29 years: 6%, 30 - 34 years: 11%, 35 - 39 years: 28.6%, 40 - 44 years: 69.3%, and 45 - 49 years: 95%. In another study covering a group of 13,351 women of Korean origin, the authors found relatively lower results, as follows: 20 - 24 years: 2.8%, 25 - 29 years: 4.5%, 30 - 34 years: 9.4%, 35 - 39 years: 26.6%, 40 - 44 years: 68.2%, and 45 - 49 years: 94.5%.

Comparing our results with the studies mentioned above, we find that between the ages of 20 - 29, the percentage of cases with low levels is similar. In the age groups between 30 - 39 years, the percentage of women with AMH < 1 ng/ml

is relatively higher in the Bulgarian ethnic group. However, in the group of women 40 years and older, this percentage ratio is lower in the women of Bulgarian ethnicity, compared to the Korean population and those coming from the territory of North America.

Another question we were looking to answer is what is the annual rate of decline of AMH in different age groups? We found that in patients aged between 25 and 29 years, the average rate of decline in AMH levels was 0.26 ng/ml/year, between 30 - 34 years, the average rate of decline in AMH levels was 0.22 ng/ml/year, between 35 - 40 years, the average rate of decline in AMH levels was 0.20 ng/ml/year, between 40 - 44 years, the average rate of decline in AMH levels was 0.13 ng/ml/year and between 44 - 50 years, the average rate of decreasing AMH levels was 0.06 ng/ml/year.

In the study by Seifer *et al.* [38], with single-year-specific median determination, the mean annual decrease in mean serum AMH was 0.2 ng/mL/year until age 35 years and then decreased to 0.1 ng /mL/year after age 35. Other authors, who included 5069 women from a region close to our population, presented a similar rate of AMH reduction [39]. This analysis in these two studies was performed on women with infertility who sought consultation and treatment at IVF clinics.

Analyzing the rate across the three studies, it is evident that the rate of decline in AMH is greater in younger women, compared to women of advanced reproductive age. The decline curve appears to be linear.

In support of this thesis, several authors suggest a linear decline of logAMH with time, until menopause. The authors included women aged between 35 and 48/mean 41/years [40] and mean age 42 years ± 2.7 years [41]. What is specific about these studies is that the groups of women were aged 40 and over, where the overall trajectory of AMH decline appeared to be more or less linear.

The concept of the linear rate of AMH decline is based on the premise that relatively high (or low) AMH levels with advancing age will remain relatively high (or low). Following this principle, a lower age-specific AMH level should therefore be associated with a shorter time to menopause [42] [43].

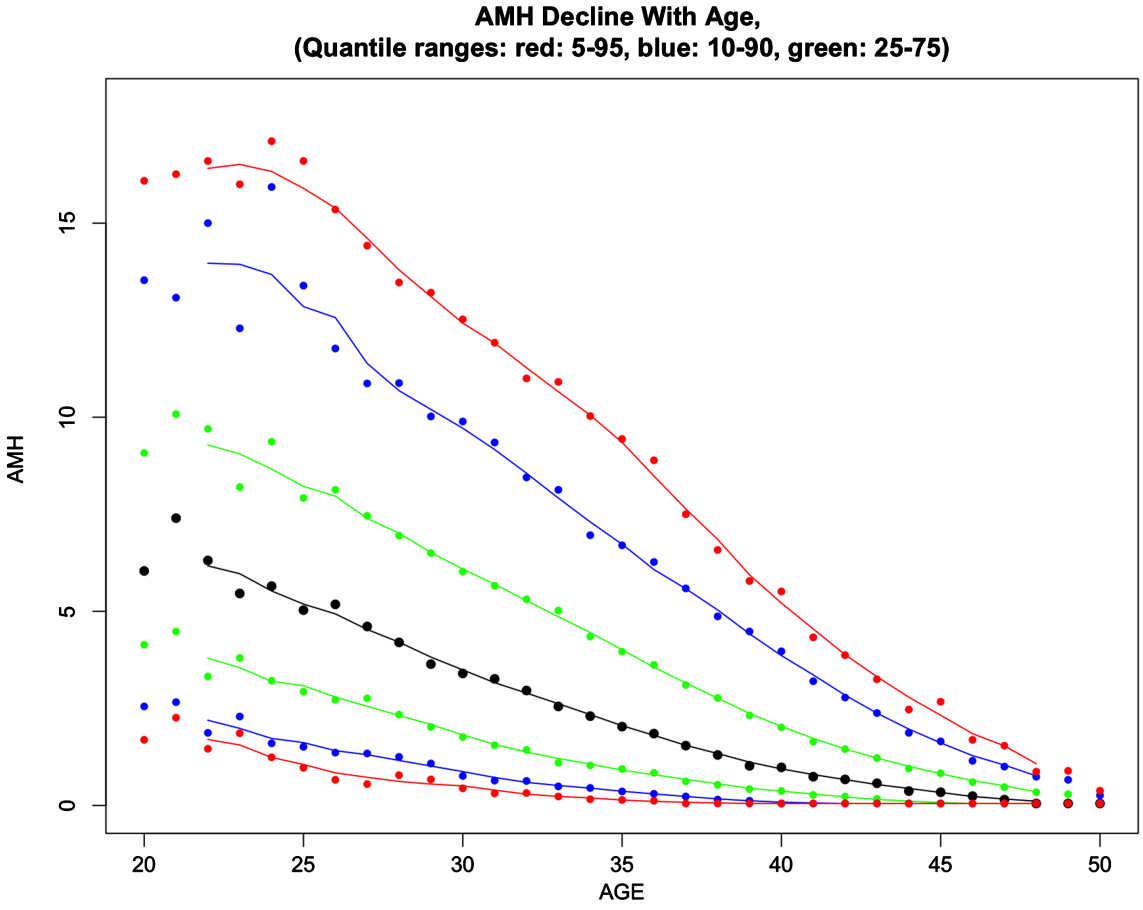
Consistent with several previous studies [44], we observed that AMH levels decline continuously with age for women of reproductive age. Zhu *et al.* [45] reported that AMH levels decreased by an average of 6.2% per year.

Several studies support the thesis of a non-linear decrease in median AMH levels with advancing age. According to Faddy *et al.*, different levels of AMH reduction are present, and the rate of decline accelerates after reaching a certain numerical threshold. The number of follicles at this threshold was initially determined to be around 25,000 [46], and it was later suggested that they differ for each individual [47]. Thilagam [48] expanded this mathematical model by including the influence of other hormones involved in reducing ovarian function. According to other authors, at higher initial AMH levels, a slower rate of AMH decline is found, but later the rate increases. This is probably due to an inhibitory effect on AMH, respectively the growth of antral follicles [49] [50] [51], a

phenomenon effective up to a certain age or ovarian reserve threshold. This change was established experimentally in a mouse study in which the decline in growing follicles and AMH levels accelerated with age [12].

In an in-depth longitudinal study, de Kat *et al.* [52] found that the curve of AMH change with age and time to menopause were not identical, because the rate of decline differed among individuals. The rate of AMH decline depends on initial AMH levels and is unrelated to age. Initial differences between women with relatively high and low age-specific AMH decreased over time. This correlates with the hormone change we found (Graphic 4). Not all women follow the same trajectory of AMH decline. The greatest differences in AMH levels between women can be found early in reproductive life, after which point they decrease. The authors found that higher baseline AMH levels were associated with a slower percentage decline in AMH over the previous year, especially for women aged 20 to 40 years.

Similar results were found by Hao *et al.* According to the authors, AMH levels are relatively stable in women in their early 20s, after that they decrease slowly in percentage terms in women aged 25 to 34 years, and the decrease is then accelerated in women over 35 years of age. When the age increased from 44 to 49 years, the AMH level dropped approximately 91.78% within 5 years, suggesting



Graphic 4. Quantile ranges in the various percentiles at different ages.

that the ovarian reserve was almost completely depleted.

Most studies analyzed median AMH levels at specific ages, namely 25, 30, 35, 40, and 45 years. Examining a Bulgarian population at these ages, we found that serum AMH levels were as follows: 25 years: 5.03 ng/ml (2.93 - 5.92), 30 years: 3.4 ng/ml (1.76 - 6.02), 35 years: 2.03 ng/ml (0.94 - 3.96), 40 years: 0.98 ng/ml (0.37 - 2.01) and, 45 years: 0.34 ng/ml (0.05 - 0.83).

Our results are close to the study of Hao *et al.*, which included 9112 women from 15 provinces of China, who were of childbearing age and were not patients of infertility clinics. However, the characteristic of the rate of decrease of AMH in different years is different compared to women of Bulgarian ethnicity.

Similar differences were found by Bleil *et al.* [36], who analyzed ethnic differences in AMH levels in women who did not have diseases and did not need infertility treatment. The authors found that in women of European origin, the change of hormone levels in different years is as follows: at the age of 25 years: 4.70 ng/ml, 30: 4.26 ng/ml, 35: 2.94 ng/ml, 40: 1.54 ng/ml, 45: 0.62 ng/ml; African-Americans aged 25 years: 2.84 ng/ml, 30: 3.14 ng/ml, 35: 2.64 ng/ml, 40: 1.69 ng/ml, 45: 0.82 ng/ml; Latinos aged 25 years: 3.26 ng/ml, 30: 2.95 ng/ml, 35: 2.04 ng/ml, 40: 1.07 ng/ml, 45: 0.43 ng/ml; women of Chinese origin aged 25 years: 3.84 ng/ml, 30: 3.44 ng/ml, 35: 2.34 ng/ml, 40: 1.21 ng/ml, 45: 0.48 ng/ml (**Figure 1**).

When analyzing the median levels of AMH in women of different ethnic groups and age, it is evident that the decrease of the hormone has a non-linear nature with advancing age.

In the analysis of median AMH levels in the above-described studies, it is

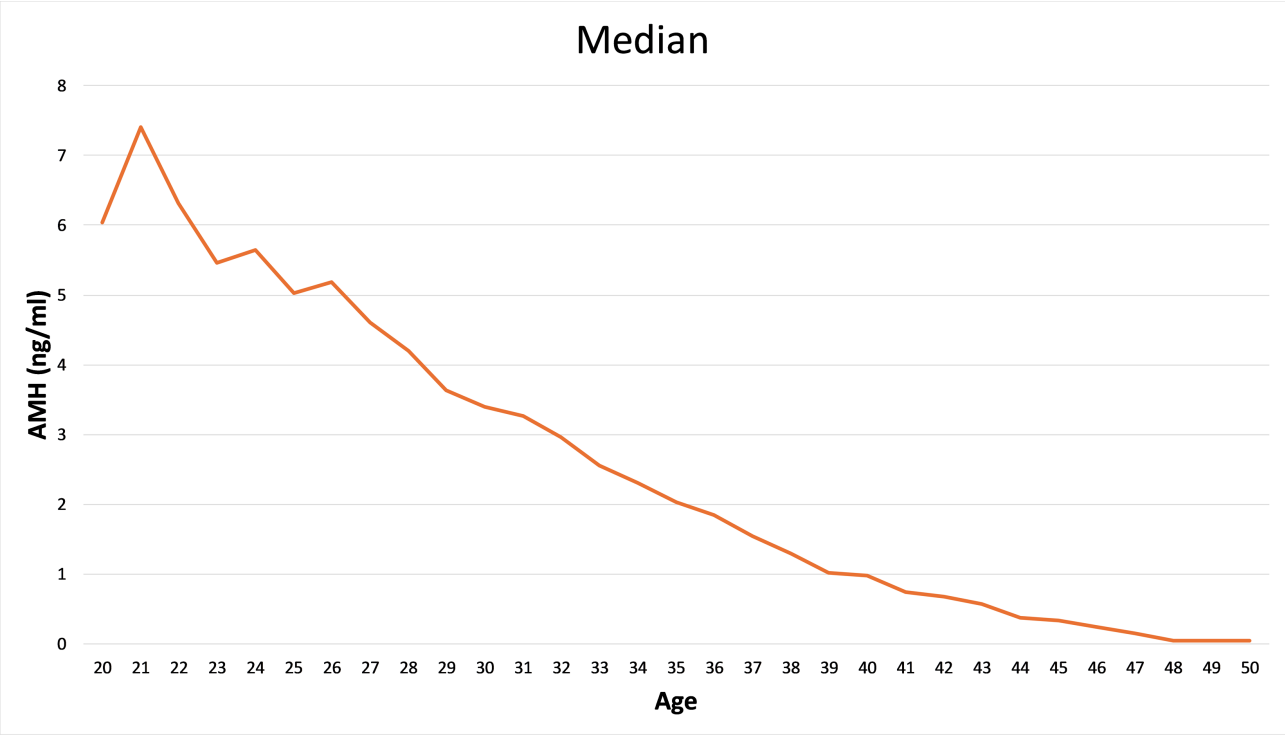


Figure 1. Distribution of single-year-specific median AMH in different age groups.

evident that the rate of AMH decline is lower between the ages of 25 and 30 years, consistent with our study. The greatest rate of decrease in AMH is between the ages of 30 and 40 in the groups of women of Chinese, Latina and Bulgarian origin, and this rate decreases between the ages of 40 and 45. However, in the groups of African-American and Caucasian women, the rate of decline in AMH increased progressively, and the greatest rate of decline occurred between the ages of 40 and 45 years. One of the reasons for the different rate of AMH decline may be the genetic differences in different groups of women. Also, when analyzing the different ethnic groups, the degree of urbanization should also be taken into account.

We hypothesize that a likely reason for these disparate results is that the rate is determined not only by the woman's age and ethnicity, but also by AMH levels. In our opinion, there are certain levels of AMH at which the rate of decline is different. From the analysis of the groups of women described above, it is evident that the rate decreases after the hormone has passed to lower levels, respectively in women aged 40 years of Bulgarian, Latino and Chinese ethnicity, the median level of AMH is between 0.98 and 1.35 ng/ml and reaching hormone levels between 0.33 and 0.48 ng/ml at age 45. Whereas in African-American and European-American women aged 40, median AMH levels were between 1.54 and 1.62 ng/ml, reaching levels between 0.62 - 0.82 ng/ml at age 45.

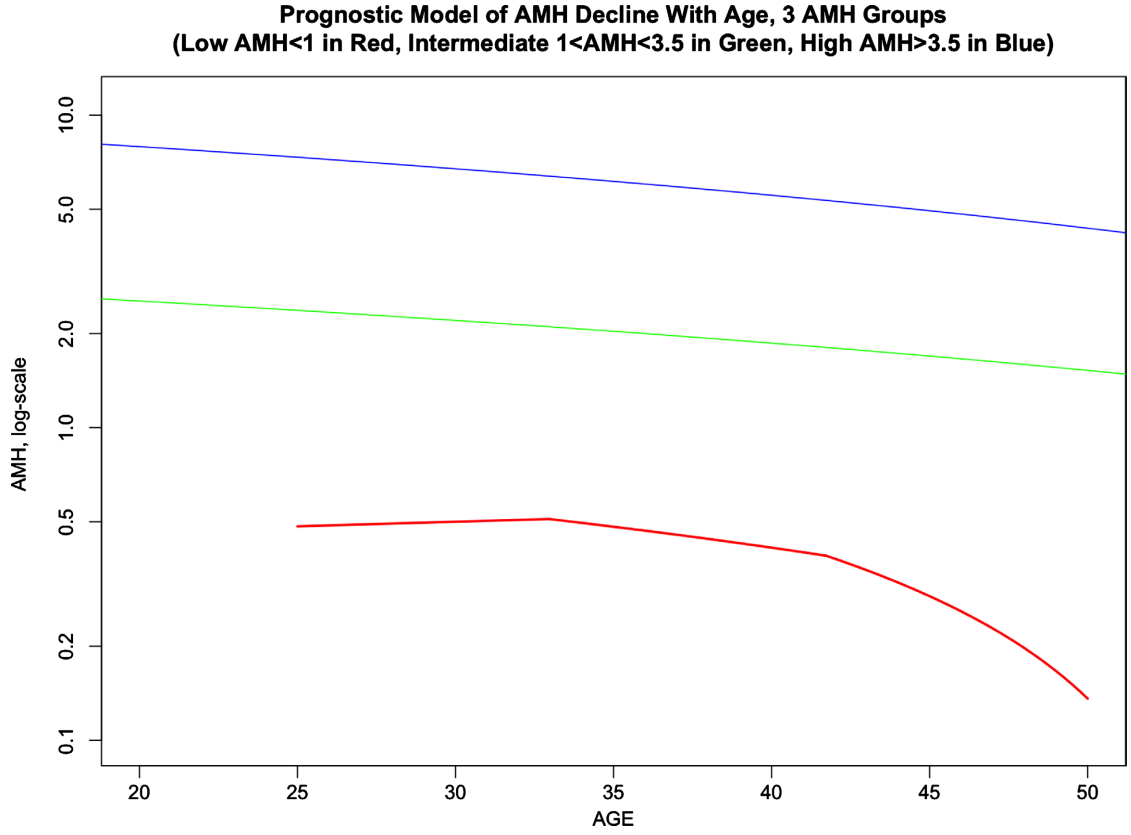
Our hypothesis that the rate of AMH change depends not only on a woman's age but also on different AMH levels is supported by additional analysis. When dividing the AMH patients into three subgroups according to the ovarian response during stimulation, as follows: high responders, normal responders and low responders, the rate of reduction of the median AMH was different and not only related to the age of the patients, but also with AMH levels. This gives us reason to assume that the rate of reduction is determined by the age of the woman and, in the case of low AMH, by certain thresholds (levels) of AMH. When we analyzed the results for the rate of AMH decline in the different subgroups, we found that: In women with AMH < 1 ng/ml, the rate of decline with advancing age changed as follows: up to the age of 33, the decline in AMH was insignificant, while between 34 and 42 years there is a rapid acceleration of the process, and after 42 years the decline of AMH is even more accelerated. This feature of AMH dynamics is the basis for explaining why it is difficult to predict when a woman will enter menopause, namely that for women with a baseline AMH level below 0.20 ng/ml, the average time to menopause is 5.99 years in the 45 to 48 age group and 9.94 years in the 35 to 39 age group [41]. This was associated with a more difficult prognosis for entering menopause in women aged 35 /C-statistic 0.68/compared to those aged 42 /C-statistic 0.93/ [42].

In women with AMH between 1 and 3.5 ng/ml, the rate of decline with age from year to year was roughly constant at 3.3%. In women with AMH > 3.5 ng/ml, the rate of hormonal decline was also approximately constant, but the annual rate of decline of 12% was significantly higher than the previous group. Based on our analysis and clinical experience, our opinion is that the rate of

AMH decline for the low AMH group has a non-linear characteristic, while in general different groups show different dynamics of AMH decline over time. Motivated by these findings, we constructed a statistical model to predict the decline in AMH levels at different ages.

Based on the observed declines with age in AMH medians across groups, we first modeled AMH dynamics in the different groups with generalized additive models that confirmed the non-linear structure of AMH decline for the low AMH < 1 group, while for the other two AMH groups (1 ≤ AMH ≤ 3. and AMH > 3.5) the dependence of the decline of AMH on age was confirmed as linear (Graphic 5). Based on these results, we constructed a two-stage prognostic model as follows: For each of the three groups, we estimated the parameters of a separate statistical model, and in the two higher AMH groups we estimated a separate robust linear model for the group, while for the low AMH group, we used a segmented robust linear model allowing for different estimated linear declines in AMH for certain age ranges. Two points of change in the rate of decline were assessed, at ages 33 and 42 years. The final Prognostic Model proceeds in 2 stages, first determining the correct AMH group of the candidate under consideration, followed by prediction with the corresponding robust model for the respective group.

With the help of the developed prognostic statistical model, it became possible to predict the individual decline of AMH at different ages of the woman. We



Graphic 5. Prognostic Model of AMH decline with ages.

found that the curves of AMH change with age are not identical, because the rate of decline differs between individuals and at different AMH levels. The rate of AMH decline depends on the initial AMH levels and varies at different ages. Thus, for different women, the rate of decrease in AMH does not follow the same trajectory. The biggest differences can be found at the beginning of reproductive life, after which this variability decreases with time.

Establishing a general model of AMH decline with age is a laborious task. In some cross-sectional studies, a quadratic decline function of AMH with age best fit the data, while others built models with polynomials or flexible splines. Consequently, the proposed relationship of AMH with age took on various forms. Some models estimated the decline of age-specific AMH levels with age to be parallel, whereas others reported converging AMH levels with higher age. With our longitudinal data, we show that the individual decline of AMH levels was closer to the latter observation, as the differences between low and high AMH levels decreased with age [53].

6. Strengths and Limitations

Our study has several strengths. Reference values of AMH were established based on a representative sample of the general population, which is the only one so far for Bulgaria and the largest described so far in the literature in terms of the percentage of examined women, relative to the population. The determination of AMH levels was performed with a single protocol and in a single laboratory. We have presented a year-by-year analysis using different percentiles of AMH values for women of reproductive age, which may be useful for use in clinical practice. On the other hand, this large sample of AMH levels provides information on the current picture of the reproductive capacity (condition) of Bulgarian women of reproductive age. A comparative analysis of AMH levels in the Bulgarian female population in two different laboratories and assessment methods was also made. We performed a comparative analysis of AMH levels in women from different countries and ethnicities.

A limitation of the present study is that it covers only a group of women of Bulgarian origin living in South-Eastern Europe. The study included women who took the test with different motives and did not exclude women with infertility, after surgical interventions on the ovary or chemotherapy, which could potentially introduce some bias for the condition in the general population [54] [55].

However, based on the results of our study, generalizations and conclusions can be made about the current state and forecasts for the reproductive capacity of women living in the Republic of Bulgaria. This study can provide basic information on the prevalence of cases with reduced ovarian reserve in women of Bulgarian ethnicity. The results of this research can help to shape public health programs to improve the effectiveness of infertility treatment in Bulgaria. The prognostic statistical model we developed would also be useful to women themselves, in terms of considering their reproductive plans. For example, if a woman

has an AMH level lower than the specific for her age, she may have a higher risk of premature ovarian aging and shorter reproductive life, and this also suggests that she may need to do early fertility planning before the ovarian reserve is depleted.

On the other hand, the prognostic statistical model may facilitate the application of AMH in clinical practice. Determining the levels of AMH, which is one of the main markers related to ovarian reserve, will enable the identification of women at higher risk who need additional support for the treatment of infertility. Determining AMH levels provides useful information that clinicians would use to determine initial stimulation doses, predict ovarian response, and achieve pregnancy as a result of IVF. Women with normal, age-specific AMH levels generally have better IVF results. Those with a lower level may have a lower ovarian response and a lower number of oocytes retrieved, while those with higher levels may have a higher risk of developing ovarian hyperstimulation syndrome (OHSS).

7. Conclusion

In conclusion, we evaluated the serum level of AMH in Bulgarian women visiting two of the largest clinical laboratories in the country. We established age-specific AMH percentile reference values based on a large representative sample. We have developed a prognostic statistical model that can facilitate the application of AMH in clinical practice and the prediction of reproductive capacity and population health. This research and analysis is aimed at professionals and all interested parties in the public, who can use the information provided to improve their understanding of fertility care. The results of this study may help shape future public health programs to improve the effectiveness of infertility treatment.

Supplementary Materials: NA

Author Contributions: M.V.: Conception, writing, review & editing, visualization. A.K.: Acquisition of data, design. E.G.: Analysis and interpretation of data, writing, review & editing, visualization. M.B.: Acquisition of data, design; S.I.: Acquisition of data; A.A.: Acquisition of data; D.T.: Design, drafting of article, funding acquisition; N.K.: Acquisition of data, design; I.K.V.: Design, conception, analysis and interpretation of data, drafting of article, writing, review & editing, supervision.

All authors approved the final manuscript. All authors contributed to the article and approved the submitted version.

Data Availability Statement

The data are available upon reasonable request to the corresponding author.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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