CLINICAL APPLICATIONS OF ANTI-MULLERIAN HORMONE AND ITS MEASUREMENT IN REPRODUCTIVE MEDICINE AND WOMEN'S HEALTH

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CLINICAL APPLICATIONS OF ANTI-MULLERIAN HORMONE AND ITS MEASUREMENT IN REPRODUCTIVE MEDICINE AND WOMEN'S HEALTH

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EDITORIAL
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Editorial: Clinical Applications of Anti-Mullerian Hormone and Its Measurement in Reproductive Medicine and Women's Health

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Keywords: Anti-Mullerian hormone, granulosa cells, disorders of sexual development (DSD), polycystic ovary syndrome, assisted reproduction, fertility prediction, ovarian insufficiency, fertility preservation

Editorial on the Research Topic

Clinical Applications of Anti-Mullerian Hormone and Its Measurement in Reproductive Medicine and Women's Health

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Anti-Mullerian hormone (AMH) is a member of the transforming growth factor-beta superfamily, which was originally identified as the factor responsible for mediating regression of the Mullerian duct in the male foetus. In the past two decades, a vast number of studies have explored its expression and roles in the adult female. It is now recognised as an important intra-ovarian regulator, being synthesised exclusively in granulosa cells of the ovarian follicles in the adult female and acting as a gate-keeper of follicular activation, growth and steroidogenesis. Its concentration in serum can now be measured using several commercially available assays and can serve as a biomarker of the functional ovarian reserve representing the follicular pool available for recruitment at any one time.

More than twenty AMH assay methods and platforms are now available commercially, with the few most commonly used platforms exhibiting good correlations between them. However, the assays frequently differ in their numerical calibration, with no international AMH standard available to harmonise the calibration, although this continues to be under active investigation. This problem of the lack of standardisation across AMH assays is a major factor limiting establishment of threshold cut-offs for various clinical applications at the moment and is reviewed by Li et al.

While serum AMH concentrations change with the age of the woman, with a general trend of decline as ovarian aging progresses, the age-specific profile of AMH also differs between ethnicities. Studies reviewed by Kotlyar and Seifer generally found higher age-specific AMH concentrations in Caucasians compared with African-American Black and Hispanic women, as well as Chinese women above the age of 25 years. Such ethnic specificity should be taken into consideration when considering clinical outcomes, particularly if ethnic specific differences in that outcome exist.

Recently, it has been recognised that AMH exists in multiple molecular isoforms, including pro-AMH, $AMH_{N,C}$, AMH_N and AMH_C. The relative abundance of the various isoforms differs between similar-sized follicles of the same woman, and even between the follicular fluid and granulosa cells within the same follicle, as reported by Mamsen et al. This poses a further potential challenge in the

development of antibody-based immunoassays to detect all molecular isoforms and in establishing which are biologically active.

As for clinical utilities, the original identification of AMH as a hormone produced from the Sertoli cells of the male foetus and during early childhood identifies the utility of measuring serum AMH in conjunction with testosterone in the differential diagnosis of disorders of sexual differentiation. This aspect of AMH clinical chemistry is comprehensively reviewed and elaborated by Josso and Rey, providing a useful guide for clinical management for these conditions.

AMH, as a biomarker of the functional ovarian follicle pool, with good correlation with the sonographic antral follicle count (2-9 mm), potentially serves as a useful diagnostic tool differentiating between the various causes of anovulation, as reviewed by Capuzzo and La Marca. In particular, women with polycystic ovary syndrome (PCOS) typically have significantly higher serum AMH concentrations in line with their higher antral follicle count, while those with WHO type 3 anovulation have very low or undetectable serum AMH. Establishment of universal diagnostic cut-offs, however, is currently limited by the variability between different assay methods which remains to be solved. The role of exaggerated AMH production in the deregulation of granulosa cells, a major factor contributing to the pathogenesis of PCOS, is further elaborated by Dewailly et al.

On the other hand, the most common clinical utilisation of serum AMH measurement is for predicting ovarian response in women undergoing assisted reproduction treatment, as reviewed by Li and Nelson. Ovarian stimulation protocols can be tailored to the predicted ovarian response for individual women, although AMH has limited predictive performance for livebirth. It is suggested that AMH, used as a continuous measure in conjunction with other prognostic factors, will continue to contribute to more refined treatment algorithms.

As a biomarker of ovarian reserve, AMH has also been explored as a predictor of natural fecundability of the female. Lin et al. presented a systematic review and meta-analysis of 11 studies including 4,388 women, indicating that serum AMH concentration has poor performance in predicting natural conception.

Upon reaching the end of the reproductive span, serum AMH declines and approaches undetectable levels. While the AMH trajectory of individual women may provide some clue as to her reproductive span, the prospective prediction of timing of menopause by serum AMH in the general population is generally too imprecise to be clinically meaningful from available studies, as reviewed by de Kat et al. In the specific scenario of women undergoing cancer treatment, measuring serum AMH pre-treatment may offer some prediction of long-term ovarian function after chemotherapy, and post-treatment AMH may offer prediction of the likelihood of gonadal function recovery, as reviewed by Anderson and Su. This may impact on treatment plans, although there is yet limited data regarding preservation of fertility post-treatment.

Looking into the future from a therapeutic angle, Rodgers et al. discuss the possibility of administering AMH as a therapeutic agent for inhibiting follicular activation and hence protecting the ovarian reserve in females undergoing chemotherapy, as well as inducing regression of ovarian cancer cells that express the AMH receptor (AMHRII). These novel therapeutic targets have been tested in animal studies with some promising results, although no clinical trials in human are yet available.

These articles in this Research Topic series provides a contemporary and comprehensive collection of current knowledge and views about the clinical utilities and limitations of AMH measurement in various aspects of reproductive medicine and women's health, as well as the potential clinical implications and applications of the AMH molecule itself. This should prompt further work to overcome the current diagnostic pitfalls and limitations, refine clinical algorithms, and establish novel therapeutic targets related to this fascinating molecule.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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High Variability of Molecular Isoforms of AMH in Follicular Fluid and Granulosa Cells From Human Small Antral Follicles

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Anti-Müllerian hormone (AMH) is a member of the TGF-β superfamily produced by follicular granulosa cells (GCs) in women from late gestation to the end of reproductive life. AMH is thought to inhibit aromatase (i.e., CYP19) expression and decrease the conversion of androgens to oestrogens, especially in small antral follicles before dominance is achieved. Thus, AMH acts as a gatekeeper of ovarian steroidogenesis. However, the exact function and processing of AMH has not been fully elucidated. The present study measured and determined AMH isoforms in human follicular fluid (FF) from small antral follicles and in human GCs using four ELISAs, western blot, and immunofluorescence analysis. We evaluated the presence of the following isoforms: full-length AMH precursor (proAMH), cleaved associated AMH ($AMH_{N,C}$), N-terminal pro-region (AMH_N), and active C-terminal (AMH_C) AMH. A negative correlation between follicle diameter and the AMH forms was detected. Moreover, western blot analysis detected various AMH forms in both FFs and GCs, which did not match our consensus forms, suggesting an unknown proteolytic processing of AMH. The presence of these new molecular weight isoforms of AMH differs between individual follicles of identical size in the same woman. This study detected several AMH forms in FF and GCs obtained from human small antral follicles, which suggests that intrafollicular processing of AMH is complex and variable. Thus, it may be difficult to develop an antibody-based AMH assay that detects all AMH isoforms. Furthermore, the variability between follicles suggests that designing a recombinant AMH standard will be difficult.

Keywords: anti müllerian hormone, human follicular fluid (HFF), granulosa cells (GCs), endocrine function, AMH isoforms

INTRODUCTION

Anti-Müllerian hormone (AMH) is a member of the TGF-β superfamily produced by human ovarian granulosa cells (GCs) in women from late foetal life to the end of reproductive life. In males, AMH is produced from early gestation by Sertoli cells in the testes and ensures regression of the Müllerian ducts. Circulating levels of AMH in males decrease at puberty (1–4).

Functionally, AMH is an important inhibitor of primordial follicle recruitment and reduces the sensitivity of preantral follicles to FSH-dependent selection for growth in mouse knockout studies (5, 6). In contrast, AMH enhances follicle growth both in size and numbers in cultured preantral rat follicles (7), suggesting that AMH acts as a growth factor for small growing follicles. However, the regulatory function of AMH in monoovulatory species may be different from that in polyovulatory species, since AMH does not affect the primordial follicle recruitment rate in sheep (8). In humans and primates, AMH may promote primary and secondary follicle growth *in vitro* either by enhancing recruitment and survival and/or by enhancing follicle growth; however, later in folliculogenesis, AMH seems to inhibit antral follicle formation and dominant follicle selection (9–12). Another *in vitro* study found that AMH suppressed initiation of primordial follicle growth in human ovarian tissue (13). Taken together, the mechanisms and functions of AMH in follicular recruitment and development may differ between species and may exert species-specific effects.

Another suggested function of AMH is inhibition of the aromatase (i.e., CYP19) expression leading to a decrease in the conversion of androgens to oestrogens in follicles prior to final selection. Intrafollicular AMH concentrations are especially high in small antral follicles below a diameter of 8 mm and may reach a concentration a thousand times higher than those in circulation (14). One function of AMH may act as a gatekeeper of ovarian steroidogenesis to ensure that small follicles are relatively quiescent; this would allow a direct ovarian/pituitary dialog regulating the development of the selected dominant follicle (15–19). Culturing human granulosa-lutein cells has shown that AMH suppresses the expression of both aromatase and P450 cholesterol side-chain cleavage enzyme (P450scc), another key enzyme in steroidogenesis at both the gene and protein levels, supporting the theory that AMH may inhibit steroidogenesis (17, 20).

Furthermore, polymorphisms in both the *AMH* gene and the AMH type II receptor (*AMHR2)* gene are associated with higher serum oestradiol concentrations in women, supporting the hypothesis that AMH is involved in the regulation of GC steroidogenesis (18).

AMH is synthesized as an inactive disulfide-like 140-kDa dimeric precursor (proAMH), comprising two 70-kDa monomers, each containing an N-terminal pro-region (AMH_N); this is important for extracellular transport and the C-terminal (AMH_C), which becomes bioactive upon proteolytic cleavage and undertakes receptor binding (21, 22) (**Figure 1**). The cleaved form consists of the C-terminal noncovalently associated with the N-terminal ($AMH_{N,C}$), which is bound to AMHR2 (22, 23) (**Figure 1**). Additional proteolytic cleavage of the $AMH_{N,C}$ complex leads to dissociation of AMH_N and AMH_C, with the AMH_C remaining biologically active and inducing intracellular SMAD signalling upon receptor binding (**Figure 1**) (23). ProAMH has two known cleavage sites: at amino acid (aa) 451 (between the AMH_N and

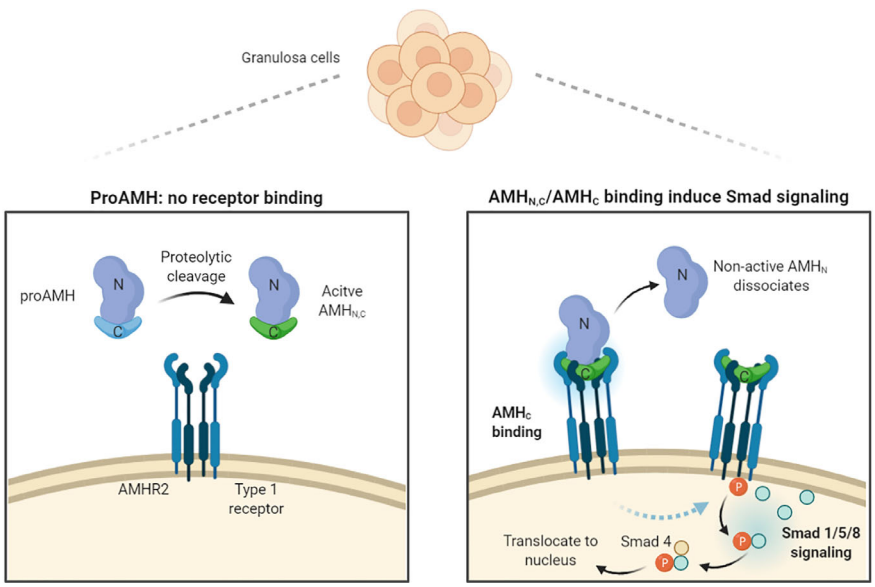

FIGURE 1 | Schematic illustration of AMH processing, receptor binding, and induction of intracellular signals. AMH is produced by GCs as an inactive promature precursor (proAMH) that is not receptor competent. Proteolytic cleavage of proAMH generate a noncovalently associated form ($AMH_{N,C}$) that can bind to the specific AMH receptor 2 (AMHR2). Upon binding to the pro region (AMH_N) dissociates. Both $AMH_{N,C}$ and AMH_C are capable of activating receptors. AMHR2 and the common Type 1 receptor phosphorylate Smads 1, 5, and 8, which bind to Smad 4, enter the nucleus and turn on AMH-responsive genes. Illustration modified from Josso (23) and created with BioRender.com.

AMH_C domains) and at aa 229 in the AMH_N region, giving rise to additional cleavage products (21, 24–26).

In contrast to other members of the TGF-β family, the N-terminal of cleaved associated $AMH_{N,C}$ enhances the bioactivity of the C-terminal and intracellular SMAD signalling (21, 22, 27). The high concentration of AMH in FF and GCs from small antral follicles, which is almost a thousand times higher than that in circulation (19, 28), allows direct use of FF and GC in western blot analysis without any prior immunoprecipitation of AMH to avoid protein overload on the gels. This is in contrast to other previous studies that included an AMH purification step prior to western blot (27, 29). The molecular isoforms of AMH present in human FF and immature GCs have not yet been studied in detail. Moreover, it is not known whether the molecular isoforms of AMH present in FF and GCs differ between follicles within the same woman or among follicles with different diameters.

The aim of the present study was to determine the molecular isoforms of AMH present in human FFs from small antral follicles and in corresponding GCs. The study used four novel high-affinity enzyme-linked immunosorbent assays (ELISAs) to detect specific epitopes on either the AMH_C or the AMH_N form in human FF. Furthermore, FF and GC samples were analyzed using western blotting with antibodies specific to AMH_C and AMH_N and immunofluorescence analysis to determine the intrafollicular expression of AMH isoforms.

MATERIALS AND METHODS

Patients

This retrospective study included a total of 41 female adolescents and women aged 15 to 38 years (mean age: 29.7 years) who underwent ovarian tissue cryopreservation (OTC) in 2006–2020. All patients gave informed consent to participate, and ethical approved was obtained from the Scientific Ethical Committee for the Capital Region [KF (01) 170/99].

Human FFs and GCs

FFs and GCs were aspirated with a 1-ml syringe and 26 G needle (Becton Dickinson, Brøndby, Denmark) from follicles visible on the surface of the ovary (3–12 mm in diameter) in connection with OTC for fertility preservation (30, 31). FFs and GCs from individual follicles were handled separately, and no samples were pooled. Follicle diameter was calculated based on the aspirated volume assuming a spherical follicle (32). Follicular fluids were centrifuged at 300*g* for 2 min at room temperature to separate GCs from FF. The GCs were washed twice in PBS, and both FF and GCs were snap frozen in liquid nitrogen and stored at -80°C for further analysis.

AMH ELISA

FFs from a total of 44 small antral follicles with a mean diameter of 6.7 mm (range: 3–11 mm) obtained from a total of 26 women were included for ELISA analysis. Four AMH ELISAs were used, each employing a specific set of antibodies that recognized different AMH sites. The AMH ELISAs used the same standardized recombinant human AMH (cat.: BA047, Ansh Labs, LLC, Webster, TX, USA) calibrators to ensure consistency between assays. ELISA analyses were performed using Ansh Labs monoclonal antibody assays against linear epitopes located on the proAMH, $AMH_{N,C}$, AMH_N, and AMH_C regions of AMH. AMH assays AL-124 (24/32) detects proAMH and $AMH_{N,C}$ (25) (**Table 1**). Assays AL-125 (24/37) and AL-145 (10/24) detected proAMH, $AMH_{N,C}$, and AMH_N (**Table 1**). AL-133 (32/33) detected proAMH, $AMH_{N,C,}$ and AMH_C (**Table 1**). The known proteolytic processing of AMH [i.e., cleavage at amino acid positions 451 and 229 (20, 24, 26)] introduces conformational changes, which may change the affinity of different antibodies.

Epitope Mapping

The specificity of the AMH antibodies was investigated by assessing their binding to 80 overlapping biotinylated peptides across the precursor AMH molecule (http://www.prot.org/UniProt/P03971) as described previously (25). The peptides were synthesized by Mimotopes Pty Ltd. (Clayton, Victoria, Australia), each 12 aa long with an overlap of 4 aa; a detailed description has previously been published (33).

Tissue Processing and Immunofluorescence Staining

One 5 mm antral follicle located in the medulla tissue was included for immunofluorescence analyses. The follicle was isolated and fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, cut into 5-µm serial sections, and processed for immunofluorescence staining. Sections were deparaffinated in xylene and rehydrated in ethanol followed by antigen retrieval in citrate buffer (10 mM sodium citrate, pH 6). Nonspecific binding was inhibited with 1% bovine serum albumin (BSA) (Sigma Aldrich, Copenhagen, Denmark). Sections were incubated with primary antibodies overnight at 4°C. Primary monoclonal mouse antibodies were specific against AMH_C and AMH_N (1:100, AMH-39/29 and 1:100, AMH-39/48, respectively, Ansh Labs, LLC, Webster, TX, USA). Sections were washed three times for 10 min in TBS with Tween 20® before incubation with goat anti-mouse IgG Alexa 488 secondary antibody (1:1,000, cat.: A11029, Invitrogen, Taastrup, Denmark). Both universal negative control serum® (BioCare Medical, CA, USA) and antibody dilution buffer were used in place of primary antibody as negative controls and showed no staining; data not shown. The monoclonal antibodies were screened on AMH_C (BA-048, Ansh Labs, LLC, Webster, TX, USA), AMH_N (BA-049, Ansh Labs, LLC, Webster, TX, USA), $AMH_{N,C}$ complex (BA-047, Ansh Labs, LLC, Webster, TX, USA), and rat AMH (BA053, Ansh Labs, LLC, Webster, TX, USA) as previously described (25).

TABLE 1 | AMH isoforms detected by the four different ELISA assays.

AMH forms	24/32	24/37	10/24	32/33
ProAMH	✓	✓	✓	✓
$AMH_{N,C}$	✓	✓	✓	✓
AMH_N	×	✓	✓	×
AMH_C	×	×	×	✓

Western Blot

Western blot analyses were performed according to the manufacturer's instructions (Invitrogen provided by Thermo Fischer, Hvidovre, Denmark). FF samples were diluted 1:1 in nuclease-free water (cat.: AM9906, Thermo Fisher, Hvidovre, Denmark), and each lane was loaded with 1 µl of FF. GC samples from individual follicles were lysed in 20 µl of radioimmunoprecipitation assay (RIPA) buffer (cat.: R0278, Sigma-Aldrich, Brøndby, Denmark) prior to analysis. GCs from one follicle were used for up to four lanes. Samples were prepared with NuPAGE LDS 4X Sample Buffer (cat.: NP0008, Thermo Fisher, Hvidovre, Denmark) and dH_2O. Dithiothreitol (DTT) was used as a reducing agent (cat.: NP0009, Thermo Fisher, Hvidovre, Denmark). Both reduced and nonreduced samples were heated at 95°C for 5 min before proteins were separated on a NuPAGE® 4–12% Bis-Tris mini gel using NuPAGE® MES SDS running buffer (cat.: NP0002, Thermo Fisher, Hvidovre, Denmark) A SeeBlue® Plus2 prestained standard marker was used (cat.: LC5925, Thermo Fisher, Hvidovre, Denmark) with the following determined proteins: myosin (188 kDa), phosphorylase (98 kDa), BSA (62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin red (17 kDa), lysozyme (14 kDa), and aprotinin (6 kDa). The 188 kDa band hardly transferred to the membrane. The proteins were subsequently blotted to a PVDF membrane (Thermo Fisher, Hvidovre, Denmark). The membrane was blocked in 5% skim milk and incubated with primary antibody (AMH-39/29, 1:5,000; AMH-39/48, 1:5,000, Ansh Labs, LLC, Webster, TX, USA) overnight at 4°C and subsequently incubated with secondary horseradish peroxidase-conjugated goat-anti-mouse antibody (1:5,000, Sigma-Aldrich) for 1 hour at room temperature. Signals were detected with Pierce™ SuperSignal West Femto Substrate (Thermo Fisher) and visualized with the DNR MicroChemi 4.2 bioimaging system.

The specificity of the AMH antibodies used for western blotting has been tested (33). Surplus recombinant AMH administered together with the primary antibody resulted in the disappearance of all AMH-related bands (33). A recombinant AMH standard was used as a positive control (cat.: BA047, Ansh Labs, LLC, Webster, TX, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0.0 (GraphPad Software, Inc., CA, USA). A linear regression model was used to determine associations between follicle diameter concentrations of AMH forms. Observations with missing values were excluded from the regression analysis. Significance was defined as $p < 0.05$.

RESULTS

Levels of proAMH, $AMH_{N,C}$, and AMH_N Correlated Negatively With Follicle Size

A significant negative correlation between follicle diameter and AMH was detected with assays 24/32 ($p = 0.007$, $R^2 = 0.159$), 24/37 ($p = 0.007$, $R^2 = 0.171$), and 10/24 ($p = 0.006$, $R^2 = 0.1735$), whereas no correlation was detected with assay 32/33 ($p = 0.079$, $R^2 = 0.077$). Assay 24/32 detected proAMH and $AMH_{N,C}$, whereas assays 24/37 and 10/24 detected proAMH, $AMH_{N,C}$ and AMH_N (**Figure 2** and **Table 1**). Assay 32/33 detected proAMH, $AMH_{N,C,}$ and AMH_C and is the only assay that captured and detected on the C-terminal. The AMH concentrations measured with the four different assays are presented in **Supplementary Table 1**, together with means, range and SD.

Increased Expression of AMH_N and AMH_C in Cumulus Cells Surrounding the Oocyte

The cumulus cells cytoplasm in a small antral follicle (5 mm in diameter) expressed both AMH_C and AMH_N (green) (**Figures 3A, B**). The intensity of the staining increased toward the oocyte (**Figure 3A**). Autofluorescence was detected in blood vessels (**Figure 3A**) (34). Furthermore, a faint signal was detected in the theca interna. Nuclear staining was given pseudocolour red (**Figures 3A, B**).

AMH Cleavage Products in FFs Did Not Change With Increasing Follicle Size

AMH cleavage products were detected using western blot analysis in FF obtained from follicles of increasing size (3.4 to 12.0 mm in diameter) from different women (**Figure 4A**). Antibodies specific for epitopes on AMH_N (i.e., AMH-39/48, Ansh Labs, LLC, Webster, TX, USA) and AMH_C (i.e., AMH-39/29, Ansh Labs, LLC, Webster, TX, USA) were used alone and together. A similar expression pattern was seen irrespective of follicle size, with bands detected at 125, 110, 87, and 46 kDa in nonreducing conditions. The 110 kDa band was more pronounced in FFs from 4.6 to 9.1 mm follicles (**Figure 4A**, dotted box). The recombinant AMH standard was detected at 23 and 100 kDa, which were different from the bands detected in FFs.

Different AMH Cleavage Products Were Detected in GCs Obtained From Follicles With Increasing Size

AMH bands of different molecular weights were detected in GCs obtained from follicles with increasing diameters from different women (**Figure 4B**). Greater expression of high molecular weight bands was found in GCs from the smallest follicles (i.e., 4.2–5.9 mm) detected with the AMH_N recognizing antibody AMH-39/48 (**Figure 4B**, dotted box). Furthermore, when using the antibody specific to $AMH_{C,}$ different cleavage products were detected in GCs obtained from follicles of increasing size (4B, arrows) during nonreducing conditions. Antibodies specific for epitopes on AMH_N (AMH-39/48) and AMH_C (AMH-39/29) were used separately.

Various AMH Cleavage Products Were Detected in FFs and Associated GCs From the Same Woman

Various AMH cleavage products were detected in FFs and associated GCs using antibodies specific to AMH_N (i.e., AMH-39/48) and AMH_C (i.e., AMH-39/29) (**Figure 4C**). These data

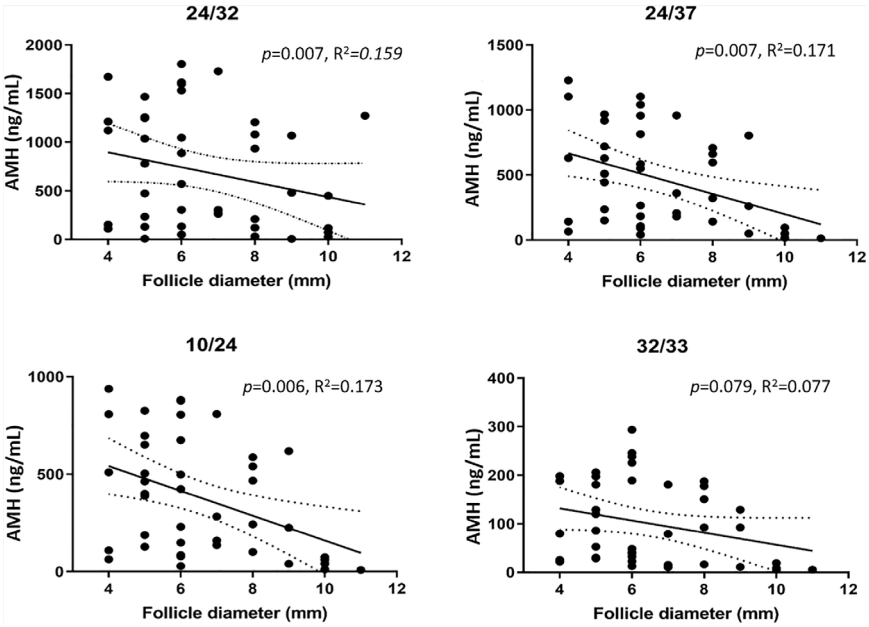

FIGURE 2 | AMH concentrations measured with four different ELISAs in FFs from small antral follicles in relation to follicle size (4-11 mm). A significant negative correlation between AMH concentration and follicle size was detected with assays 24/32, 24/37, and 10/24 (Ansh Labs, LLC, Webster, TX, USA). The solid line indicates the mean AMH concentrations in relation to follicle diameter. Dotted lines indicate 95% confidence intervals.

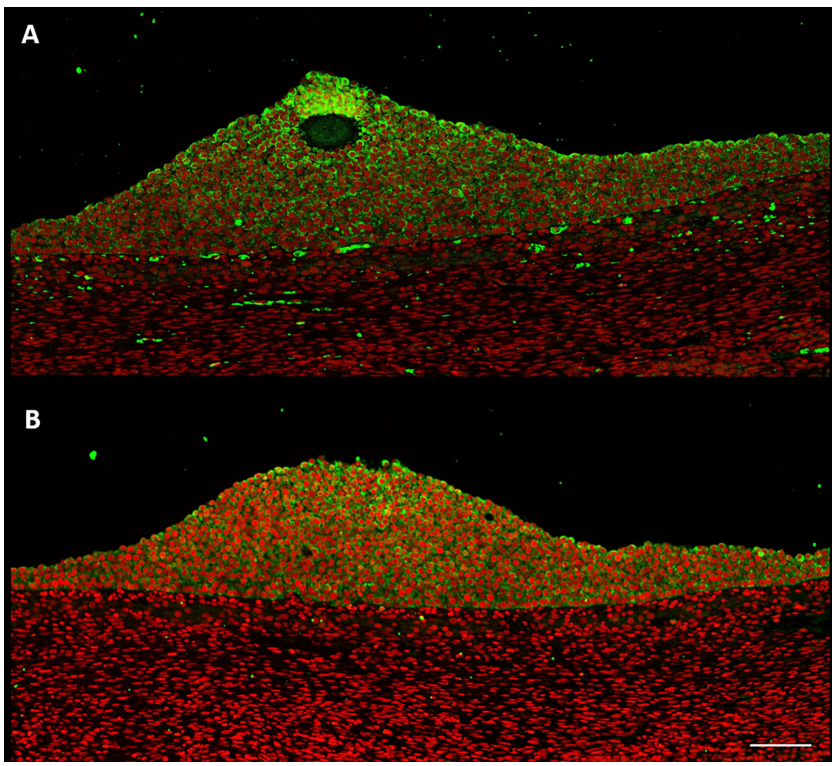

FIGURE 3 | Human small antral follicle (5 mm). **(A)** AMH_C (green) is expressed in cumulus cells with increasing intensity toward the oocyte. Nucleus (red). **(B)** AMH_N (green) is expressed in cumulus cells close to the oocyte. Scale bar: 100 µm.

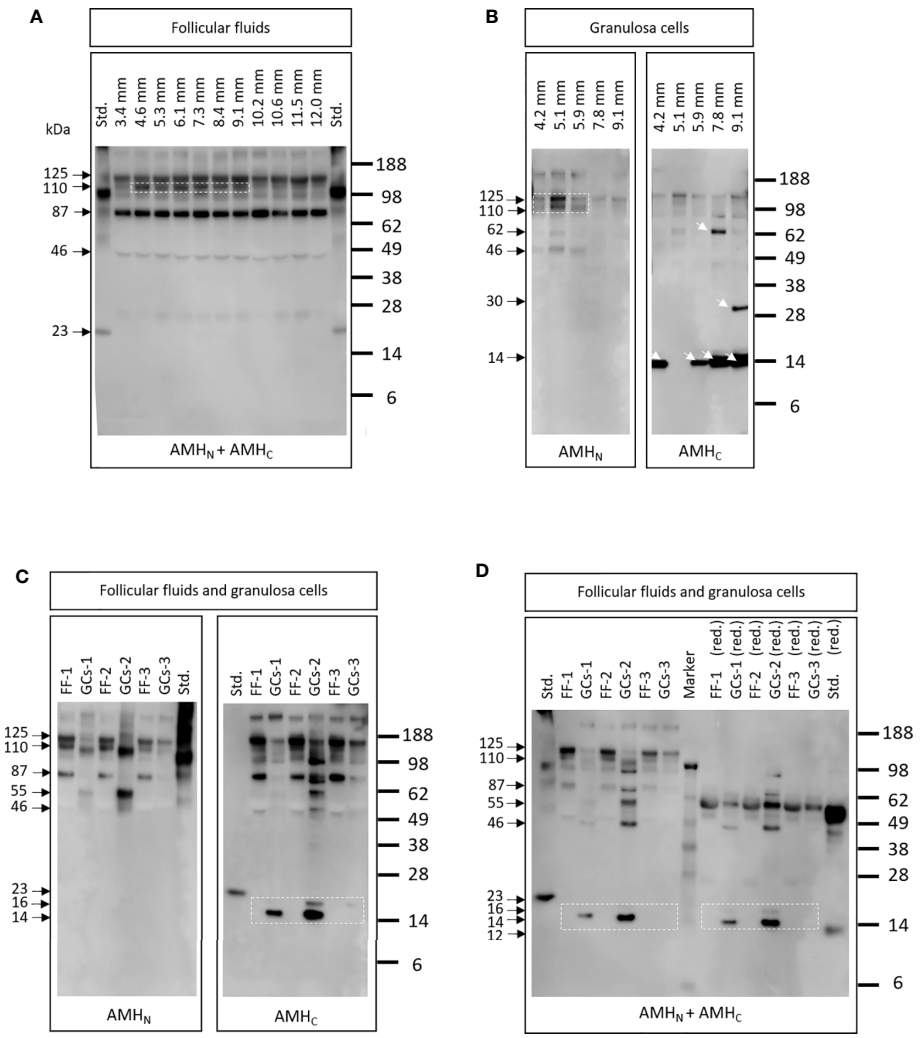

FIGURE 4 | **(A)** AMH cleavage products detected in FFs obtained with increasing diameter (3.4 to 12.0 mm) from different women. Antibodies specific for epitopes on AMH_N and AMH_C were used (i.e., AMH-39/48 and AMH-39/29, respectively). A similar expression pattern was detected irrespective of follicle size, although the 110 kDa band was more pronounced in FFs from 4.6 to 9.1 mm follicles (white dotted box). Std.: 10 ng recombinant AMH standard (Ansh Labs, LLC, Webster, TX, USA). **(B)** AMH cleavage products in GCs from follicles with increasing diameter obtained from different women. Different AMH cleavage products were detected in the GCs of different follicles (white arrows). The dotted box highlights a greater expression of high molecular weight bands in GCs from the smallest follicles (i.e., 4.2-5.9 mm). Lanes are marked with the diameter of follicles where the GCs were obtained. **(C)** AMH cleavage products detected in FF and in GCs from the same follicles. Antibodies specific to AMH_N and AMH_C were used separately (i.e., AMH-39/48 and AMH-39/29). FF-1 and GG-1 are from the same follicle etc. FF-1 and FF-2 were from the same women, illustrating that different AMH forms were detected in FF and GCs from the same follicle. Note that the AMH_C-specific antibody detected low molecular weight bands at 16 and 14 kDa in some GCs, which is not seen in FF (dotted box). Std.: 10 ng recombinant AMH standard (Ansh Labs, LLC, Webster, TX, USA). **(D)** Samples were the same as in **(C)**. Antibodies specific to AMH_N and AMH_C were used together (i.e., AMH-39/48 and AMH-39/29). Samples marked (red.) indicate that samples were treated with dithiothreitol. Low Mw bands of 16 and 14 kDa were only detected in GCs (dotted boxes) and were different from the recombinant AMH standard band at 23 kDa, suggesting that recombinant AMH does not reflect the forms present *in vivo*.

clearly illustrate that within one follicle, AMH forms differ between FF and GCs and that low molecular weight fragments of 14 and 16 kDa were only detected in GCs (**Figure 4C**, dotted box). **Figure 4D** displays the results from the same samples as in **Figure 4C**. Antibodies specific to AMH_N (i.e., AMH-39/48) and AMH_C (i.e., AMH-39/29) were used simultaneously under nonreduced and reduced conditions. Under reduced conditions, high-intensity bands at 55 kDa were detected in both FFs, GCs, and standards. The low molecular weight recombinant AMH standard was reduced from 20 to 12 kDa, whereas the low molecular weight bands in GCs at 16 and 14 kDa did not change molecular weights (**Figure 4D**, dotted boxes).

DISCUSSION

The present study demonstrates that the number of AMH isoforms in FF and GCs found in human small antral follicles

are unexpectedly high, and highly variable between follicles and between FFs and corresponding GCs in a large set of samples. In addition, the distribution of AMH isoforms in GCs differs profoundly between follicles, even in follicles of the same size from the same woman. Furthermore, GCs express AMH-related peptides that, to our knowledge, have not previously been described. The ELISA data and western blot analysis show that the processing of AMH does not follow the patterns obtained with *in vitro*-produced recombinant AMH. The processing of AMH in the human follicular compartment appears to be more complex than previously described; several unknown cleavage products were detected.

In western blots, we detected bands at 125 kDa (proAMH homodimer), 110 kDa (AMH_N homodimer), 100 and 87 kDa (novel cleavage fragments), and 55 kDa (AMH_N monomer). Furthermore, bands at 16 and 14 kDa were detected in GCs, possibly representing AMH_C fragments since the staining is only seen using the antibody that recognizes AMH_C. An increased rate of proAMH cleavage was detected in 4–9 mm follicles, giving rise to a strong 110 kDa band in these FFs, indicating that the smallest follicles contribute the most to bioactive AMH (**Figure 4A**). This finding fits with the observation that CYP19 remains downregulated in these small follicles and only becomes upregulated after follicle selection at a diameter of 8–10 mm (19). The AMH_C is normally ascribed a molecular weight of approximately 25 kDa, which under reducing conditions results in a molecular form of approximately 12 kDa. This is about the same as that observed in the present study using a recombinant AMH standard. Interestingly, a prominent 14- and 16-kDa band is detected in several of the GC samples using an antibody specific to AMH_C, which furthermore is unaltered by exposure to reducing conditions (**Figure 4D**). This indicates that no disulfide bridges are present in these molecular forms, and they appear to consist of one peptide chain. It is surprising to note that different AMH isoforms present in GCs do not match those present in the corresponding FFs. The different Mw bands may be alternative cleavage products, different glycosylation forms or degradation products, caused by enzymatic catalysis in GCs and FFs (35). Alternatively, AMH_C may have an intracellular function as a transcription factor in GCs (33) and are to a lesser extent released in the FFs.

To our knowledge, this finding has not been reported in the literature and raises the question of whether these AMH-related peptides exert physiological functions. In this context, it is worth noting that we detected nuclear localization using immunohistochemical staining of cell nuclei in some follicles using the AMH_C-detecting antibody (i.e., 39/29 antibody) (33), while the AMH-39/48 antibody showed no nuclear staining. This may suggest that autocrine regulation and processing of AMH within human GCs takes place, which warrants further investigation.

These patterns of AMH peptides, detected by the two antibodies used for western blotting, demonstrate that AMH is proteolytically processed in different ways and suggest additional cleavage sites. These results, in combination with the four ELISAs employed on human FF samples from small antral follicles (in which a the same AMH standard was used), demonstrate that it will be difficult to develop a single assay to detect all AMH-related peptides in a biological sample. Furthermore, it will be difficult to produce a standard that can be used to define an AMH concentration in each sample. Previous studies have evaluated the performance of commercial AMH assays in serum and found very different performances between assays. In some assays, AMH was hardly detected. Other assays found normal concentrations of AMH corresponding to the number of follicles present (36, 37), probably reflecting that the antibodies employed captured different epitopes. It is important to note that the concentration of AMH in FFs is a thousand times higher than it is in serum (38). Furthermore, some of the AMH isoforms present in FFs and GCs may be degraded in serum, explaining why we do not expect the isoform profiles detected in the follicular compartment to be reflected in serum. Most likely, the low-Mw AMH_C isoforms detected in GCs are unglycosylated and therefore rapidly cleared in circulation. It has previously been demonstrated that proAMH and $AMH_{N,C}$ are the only confirmed isoforms present in serum, with proAMH being the dominant form (27). This suggests that assays designed for measurements in serum should preferably capture and detect at the N-terminal because this appears to be the most stable isoform. The present results suggest that a combination of two or more capturing antibodies or a single epitope two-sided antibody should be used to assure binding of as many AMH-related peptides as possible. However, a combination of antibodies targeting both the N- and C-terminal parts of AMH could potentially result in measurements of cleaved fragments and thereby cause overestimation.

The AMH_N-specific antibody detected high molecular weight AMH bands of 140, 110, and 87 kDa in the recombinant AMH standard, which were similar to the bands detected in GCs but different from the bands detected in FFs (i.e., 100 and 55 kDa). The AMH_C-specific antibody detected only one low molecular weight band in the recombinant standard of 23 kDa, whereas this antibody detected 16 and 14 kDa bands in GCs and no low molecular weight bands in FFs. Moreover, this AMH_C-specific antibody detected an 87 kDa band in FFs, which was not present in GCs, suggesting that further proteolytic cleavage takes place in FFs that does not take place in the GCs.

Additionally, high molecular weight bands of 140, 110, and 100 kDa were detected with this AMH_C-specific antibody in both FFs and GCs; the 110 kDa fragment could be an AMH_N homodimer, whereas the others may be alternative cleavage forms of AMH.

The four ELISAs, specific for a unique pair of epitopes on the AMH molecule, detected very different concentrations of AMH-related peptides. We found a significant negative correlation between the follicle diameter and the concentration of AMH isoforms measured with three of the four ELISAs, supporting previous findings (14, 19, 32). The assay capturing and detecting the AMH_C did not detect a significant correlation between follicle diameter and AMH concentration, suggesting that the contribution of AMH_C may be constant regardless of the size of

the follicle. Since the AMH_C is not glycosylated, it is likely to be cleared from circulation relatively fast, which is corroborated by its near absence in circulation (27). Thus, it may be hypothesized that AMH exerts a biological function primarily within follicles, where the concentration is much higher than that in circulation, which on the flip side suggests that AMH may not be biologically active outside of the follicle in circulation and other biological fluids, where the concentration is much lower and AMH_C is not present. This further demonstrates that AMH in FFs is present in multiple molecular forms.

Our study demonstrates that AMH expression increases in cumulus cells surrounding the oocyte, showing a gradient with lower expression in mural cells and thereby variable AMH expression per cell. This result is similar to that observed in sheep follicles (8) and suggests that the oocyte affects AMH expression in cumulus cells. In a previous study, we found that AMH remained highly expressed in cumulus cells compared to mural GCs (39), which corroborates the results of this study. The functional significance of this finding is not revealed by the study. However, it can be hypothesized that the increased AMH expression in cumulus cells may inhibit aromatase expression and thereby the conversion of androgens to oestrogens, which has been shown in sheep follicles (8). Faint AMH staining was detected in theca cells, which may represent AMH bound to AMHR2 (40). A similar observation was made in human foetal male mesonephri and explained by AMH bound to its receptor (33).

The present study has taken advantage of the high concentrations of AMH in FFs and GCs to obtain a detailed picture of the various molecular isoforms in FFs that are present in human small antral follicles. The vast majority of AMH is produced within small antral follicles, from which AMH diffuses to circulation. Thus, it is conceivable that the molecular isoforms of AMH in follicles will resemble those in circulation. However, the individual follicles may show pronounced differences, which in circulation will be the average of what is released from the pool of ovarian follicles. In addition, further proteolytic processing may take place in circulation, giving rise to additional cleavage products that may not reflect peptides within the follicles.

Taken together, these results demonstrate the complexity of predicting the biological activity of AMH detected in FFs and GCs. AMH cleavage products differ between FFs and GCs from the same follicle and between follicles with the same diameter from the same women, illustrating that processing AMH gives rise to additional cleavage products, some of which may be active and some inactive. As ovarian GCs are the main producer of AMH in women, the molecular isoforms present in circulation are likely to reflect those in FFs and GCs; therefore, it is difficult to envision an ELISA that will detect the various AMH isoforms with similar efficacy in different serum samples. This suggests that serum AMH measurements and their clinical outcomes may depend on the isoform measured by a specific ELISA. In addition, it will be difficult to define an AMH measurement protocol that will standardize the content of AMH in different samples.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Scientific Ethical Committee for the Capital Region, Kongens vænge 2, 3400 Hillerød, Denmark. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LM wrote the paper, performed the statistical analysis, obtained the FFs and GCs in connection with ovarian cortex cryopreservation, assisted in WB, prepared figures and tables, and interpreted data. JJ and JB did the IF analysis. JB, SK, and SP obtained and processed FFs and GCs. AK and BK developed the antibodies and the ELISAs used (Ansh Labs, LLC, Webster, TX, USA) and interpreted the data. EE recruited patients for the project and performed the ovariectomies. CA interpreted the data, assisted in writing the paper, and was responsible for the study design. All authors critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.617523/full#supplementary-material

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MINI REVIEW
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Challenges in Measuring AMH in the Clinical Setting

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Serum anti-Mullerian hormone (AMH) is a widely used marker of functional ovarian reserve in the assessment and treatment of infertility. It is used to determine dosing of gonadotropins used for superovulation prior to *in vitro* fertilization, as well as to determine the degree of damage to ovarian reserve by cytotoxic treatments such as chemotherapy. AMH is also now used to predict proximity to menopause and potentially provides a sensitive and specific test for polycystic ovarian syndrome. Twenty one different AMH immunoassay platforms/methods are now commercially available. Of those compared, the random-access platforms are the most reliable. However, to date there has not been an agreed common international AMH reference preparation to standardize calibration between the various immunoassays. Recently, a purified human AMH preparation (code 16/190) has been investigated by the World Health Organization as a potential international reference preparation. However, this was only partially successful as commutability between it and serum samples was observed only in some but not all immunoassay methods. Development of a second generation reference preparation with wider commutability is proposed.

Keywords: anti-Müllerian hormone, enzyme-linked immunosorbent assay (ELISA), automated chemiluminescence immunoassay, reference preparation, international standard

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INTRODUCTION

Anti-Mullerian hormone (AMH), also known as Mullerian-inhibiting substance (MIS), was first recognized in the 1940s as the factor which determines regression of the Mullerian duct in the male fetus although it was only formally characterized and cloned in the 1980s (1, 2). Its production in the adult female was first reported in 1990 (3). With the development of serum AMH immunoassays it has become apparent that AMH is a clinically useful marker of functional ovarian reserve (4, 5) and thus of clinical value in the treatment of infertility, where measuring the follicle reserve is important.

Serum AMH is now used as a diagnostic test in infertile women undergoing controlled ovarian stimulation as part of an *in vitro* fertilization (IVF) program (6) and in the assessment of polycystic ovarian syndrome (PCOS), risk of ovarian hyperstimulation syndrome, prediction of menopause

and monitoring the impact of cytotoxic chemotherapy and radiotherapy on ovarian function. Currently there are commercial kits available from more than 14 manufacturers (7, 8). However, the absence of an agreed international AMH reference preparation has resulted in confusion in defining clinical reference ranges between different kits. The aim of this report is to describe the development of AMH immunoassays and AMH reference preparations, and discuss the recently described WHO AMH Reference Reagent for immunoassay standardization.

AMH GENE AND MOLECULAR STRUCTURE

AMH is a member of the transforming growth factor-beta (TGF- ß) superfamily. It is a 140 kDa homodimeric glycoprotein consisting of two identical glycoprotein subunits linked by disulphide bonds. In humans, AMH is encoded by the *AMH* gene, which is located on chromosome 19 p13.3. The *AMH* gene is 275 bp in length and consists of five exons. The GC-rich 3' end of the fifth exon codes for the bioactive part of the AMH molecule (8, 9).

The *AMH* gene encodes a pre-protein of 560 amino acid residues (pre-proAMH) which is cleaved to produce the precursor (proAMH) (AMH_{25-560}) that has no binding to the AMH receptor. It undergoes proteolytic cleavage by subtilisin/kexin proprotein convertases to the bioactive form, $AMH_{N,C}$. $AMH_{N,C}$ is a complex consisting of the N-terminal fragment (AMH_N) and the C-terminal fragment (AMH_C) associated non-covalently. The AMH_N fragment is a 110 kDa homo-dimer formed by two 57kDa subunits, whereas the AMH_C fragment is a 25 kDa homo-dimer formed by two 12.5 kDa subunits. Only $AMH_{N,C}$ and AMH_C are bioactive on AMH receptors (**Supplementary Figure 1**). ProAMH and $AMH_{N,C}$ are the circulating forms detectable in the blood in varying ratios, whereas the free AMH_C and AMH_N are not detectable in the blood circulation in physiological conditions. Current commercially available immunoassays detect both proAMH and $AMH_{N,C}$, and the reported values are a composite of both. The physiological role of proAMH in the circulation is currently not clear (10–15).

CORRELATION OF SERUM AMH WITH OVARIAN RESERVE

Although the primordial follicle count is conceptually the definitive parameter representing the ovarian reserve in a woman, it can only be measured directly by histological examination of the whole ovary after oophorectomy. Hence, surrogate markers which correlate with the primordial follicle count have been explored for clinical use, and both the AMH measured in serum and the antral follicle count (AFC) measured sonographically have been demonstrated to serve this purpose.

One study of 42 women undergoing oophorectomy for benign gynaecological conditions revealed a significant correlation ($p<0.0001$) between serum AMH and the ovarian primordial follicle count determined histologically, both unadjusted ($r=0.72$) as well as after adjustment for chronological age ($r=0.48$); the correlation coefficients were similar between AFC and primordial follicle count (unadjusted $r=0.78$ and adjusted $r=0.53$) (16). Most of the available studies showed a high correlation between serum AMH level and AFC (17–19).

INTRA- AND INTER-CYCLE VARIATIONS OF SERUM AMH

Most studies have demonstrated small fluctuations in serum AMH across the normal menstrual cycle with a decline in the late follicular phase. This pattern has been explained by a decrease in AMH secretion from the lead follicle as it achieves dominance prior to ovulation. However, the magnitude of these intra-cycle fluctuations is small and is not generally considered clinically relevant (20–24). One study revealed that the intra-cycle fluctuations remained within the same quintile in 72% of women and crossed two quintiles in only 1% of women (22). Two prospective studies (22, 25) explored the inter-cycle variability and suggested that between-cycle reproducibility of serum AMH is higher than that of serum FSH or AFC, and that only 11% of the variability resulted from intra-individual fluctuations between cycles (intra-class coefficient 0.89).

There are a small number of situations in which the intracycle fluctuation in AMH should be taken into account when assessing ovarian reserve. In particular, late reproductive aged women have a reduced number of follicular waves through the cycle, and hence AFC may be low and serum AMH can show marked changes (26) paralleling the pattern of the follicular waves. Similarly, following chemotherapy, the antral follicle reserve may be severely reduced and serum AMH profile may vary across the cycle or treatment period. In cases where the ovarian reserve is low, the more sensitive AMH immunoassays (sensitivity ~0.1ng/ml) are needed. Depending on its application, standardising the serum collection time in the cycle would appear to be a wise prerequisite in some situations. Sample collection alongside FSH and LH in the early follicular phase of the cycle allows for standardisation of timing in such situations. If the woman does not have a natural cycle then a random sample would be acceptable.

CLINICAL APPLICATIONS

A number of clinical situations have been identified in which serum AMH can be a useful diagnostic marker.

a) Ovarian Reserve Testing

Prediction of ovarian response to superovulation is the most common application of serum AMH (6, 27). Two individual patient data meta-analyses (28, 29) have shown that both serum AMH and AFC had good performance in predicting poor

ovarian response as well as excessive response. Ovarian stimulation regimes are now individualised to provide the optimum number of oocytes while avoiding risk of severe OHSS (30). In the IVF context, low oocyte yield in "poor responder" patients inevitably results in a smaller pool of cryopreserved embryos, thereby reducing the cumulative livebirth rate (LBR) from one IVF cycle, whereas larger numbers of eggs/embryos offers a higher cumulative LBR.

b) PCOS

It is now well recognised that serum AMH is elevated in women with PCOS (5, 31, 32). In a recent study, AMH exhibited high specificity:sensitivity based on the receiver-operating characteristic (ROC) curve in predicting PCOS compared with age-matched controls (32).

c) Prediction of Menopause

Several studies (33–35) have explored the use of single or multiple AMH samples over time as a means to predict menopause. Studies by Finkelstein (35) assessed the probability of AMH predicting menopause in women in the late reproductive age (~47y) and showed that in combination with age and body mass index, AMH measurement predicted the occurrence of menopause within 12 to 36 months (area under the ROC curve = 0.88-0.99). These conclusions were derived from an ultrasensitive AMH enzyme-linked immunosorbent assay (ELISA) with a lower detection limit of <2pg/ml. Assessment in women over a longer lead time (14 years) showed an improved prediction of menopause when including knowledge of the AMH decline rate (34).

d) Monitoring the Return of Fertility in Those Women With Cancer Treated With Chemotherapy

Recovery of fertility in women following chemotherapy is a poorly defined area with evidence of shortened reproductive lifespan and infertility (3). Using AMH to monitor this process has revealed a complex pattern of recovery which is dependent both on the type of chemotherapy used and the woman's age at treatment (36–38). In the study of Su et al. (38) dried blood analyses were undertaken using validated ELISA methodology with a sensitivity of 30pg/ml. The AMH level post treatment was 140pg/ml requiring sensitive ELISAs. Only 7% of samples were undetectable in this study.

e) Serving as a Tumour Marker for Some Cancers

AMH can serve as a tumour marker for the detection or follow-up for recurrence of granulosa cell tumors (39–41).

EVOLUTION OF AMH ASSAY METHODS

Measurement of AMH in serum from adult women using ELISA was first reported in 1990 (3, 42). In the early 2000's, when clinical studies utilizing AMH measurement were initiated, two commercial AMH ELISAs were available, manufactured by Diagnostic Systems Laboratories, Inc. (DSL, Webster, Texas, USA) and Immunotech (Marseille, France). DSL and Immunotech were subsequently acquired by Beckman Coulter, Inc. with the development of a second generation ELISA under the name "AMH Gen II ELISA". This ELISA utilized the antibodies from the DSL kit and the AMH reference preparations from the Immunotech kit (43). Following its introduction in 2010, the AMH Gen II ELISA became the most widely used assay for AMH. However, its reliability was questioned due poor assay reproducibility, particularly following sample dilution and sample storage under different conditions (5, 44). The poor reproducibility was subsequently attributed to assay interference due to binding of serum complement protein C1q to the capture antibody. A pre-mixing protocol was then recommended by the manufacturer to overcome this problem. It was postulated that pre-mixing the test sample with the highly anionic buffer inactivated complement, hence reducing the interference. However, serum AMH values generated by the pre-mixing protocol are significantly higher compared with the conventional protocol (45).

More recently, additional AMH immunoassay kits have become available, including the ultrasensitive AMH/MIS ELISA kit (Ansh Laboratories, Texas, USA), the automated Access AMH kit (Beckman-Coulter Diagnostics, USA) and Elecsys® AMH Immunoassay (Roche Diagnostics International Ltd, Indiana, USA). The latter two are automated immunoassays that utilize chemiluminescence for detection and are not susceptible to interference by serum complement (46). **Table 1** lists the analytical characteristics of the common commercial AMH assays that are currently available. Additional new AMH immunoassays are presented in the recent article by Ferguson et al. (8).

TABLE 1 | Analytical characteristics of the common commercial AMH assay methods according to information from the manufacturers.

Characteristic	AMH Gen II ELISA	Access AMH assay	Elecsys AMH Immuno-assay	Ultra-Sensitive AMH/MIS ELISA	MenoCheck picoAMH ELISA
Lower limit of detection (LoD)	0.08 ng/ml	0.02 ng/ml	0.01 ng/ml	0.023 ng/ml	1.3 pg/ml
Lower limit of quantitation (LoQ) with <20% CV	0.17 ng/ml	0.08 ng/ml	0.03 ng/ml	0.06 ng/ml	3.2 pg/ml
Intra-assay coefficient of variation	<=5.4%	<=1.7%	<=2.6%	<=4.0%	<=5.5%
Inter-assay coefficient of variation	<=5.6%	<=2.8%	<=3.9%	<=4.8%	<=8.1%
Calibration points	7 points	6 points	2 points	6 points	6 points
Sample volume	20 µl	20 µl	50 µl	25 µl	100 µl
Assay time	>3 hours	40 minutes	18 minutes	2.5 hours	4.5 hours

COMPARISON BETWEEN THE VARIOUS AMH ASSAY METHODS

In the earlier years, a comparison between the Immunotech and DSL ELISAs showed widely different regression relationships, with lower, comparable or higher serum AMH values by Immnotech compared with DSL ELISA being reported in different studies (47–50).

Studies comparing the AMH Gen II ELISA, DSL and Immunotech AMH ELISAs (30, 50, 51) showed good correlations of AMH Gen II ELISA with both the DSL and Immunotech kits although higher numerical values were shown by AMH Gen II ELISA compared to the latter. A higher numerical value was generated by the AnshLabs assay compared with the Gen II assay (43, 50–52).

AMH values obtained with the Gen II kit were well correlated with those generated by the Access and Elecsys® automated immunoassay methods, with correlation coefficients being >0.9 ($p<0.001$) in all pairwise comparisons (52). Passing and Bablok regression revealed that the values generated by the Access AMH assay were comparable to those generated by the Gen II assay, whereas those generated by the Elecsys AMH Immunoassay were systematically lower (**Supplementary Figure 2**). The bias between the Beckman-Coulter platforms (Gen II assay and Access AMH Assay) and the Elecsys AMH Immunoassay was uniform across the whole range of values studied. The finding concurred with previous reports, although the Elecsys AMH Immunoassay was claimed to be standardized against the Gen II assay (53–57). This differential calibration should be kept in mind when results generated by the different assay methods are interpreted or compared in clinical practice or research settings.

ASSAY STABILITY UPON DIFFERENT SAMPLE STORAGE CONDITIONS

In the Access AMH assay and Elecsys AMH Immunoassay, serum samples frozen at -20°C and -80°C gave significantly lower AMH values ($p<0.05$) compared with freshly collected samples (52), with significantly lower values for those stored at -20°C compared with -80°C ($p<0.05$). The magnitude of the difference between immunoassays is small (<0.2 ng/ml) and may not be clinically important. The basis for this loss of immunoactivity with frozen storage remains to be explored.

DEVELOPMENT OF AN INTERNATIONAL AMH REFERENCE REAGENT FOR IMMUNOASSAY

There has long been an unmet need for an internationally available reference material for AMH. This would allow calibration of diagnostic AMH immunoassays against a standard, which would then allow values obtained from different assay systems to be compared. An international collaborative study (7, 8) was thus undertaken by the World Health Organization (WHO) to produce a reference material purified from media from a stable human ovarian cancer cell line and available in ampoules (coded 16/190), and to derive an immunopotency evaluation for this human AMH preparation. The AMH preparation consisted of the full length 140kDa form with a modification of the internal cleavage site to ensure maximum cleavage between the pro-hormone and mature forms of the molecule (58) which represent the major forms of AMH in human serum (14, 15, 32). The WHO study involved the distribution the WHO reference preparation to participant laboratories, along with 21 human serum samples of varying origin. Each participant laboratory was requested to include these samples in their own immunoassay system using the AMH reference preparation of their own kit. Study participants used 21 different assay methods, 19 of which were different methods/platforms (either manual or automated) combinations. Since there is currently no recognized AMH preparation to act as an international reference, the results for serum values and the WHO reference reagent varied markedly between assays. The immunopotencies of the WHO reference reagent for the 21 laboratories ranged from ~350 to ~1200 ng/vial. In order to develop a consensus potency for the WHO Reference reagent, results of those 16 methods which were statistically comparable were combined to yield a robust geometric mean of 489 ng/ampoule. In addition, the bias of individual serum AMH samples for all kit results from the consensus means were also determined. In parallel, the bias attributed to the WHO AMH reference reagent in individual assays from the consensus mean was determined and compared with the corresponding bias observed with the serum samples.

Interestingly, in many of the assays the bias for the WHO AMH reference reagent within assay was statistically dissimilar to that observed with serum samples. Thus, while the use of the WHO AMH reference reagent as an International reference preparation should reduce the variability between some assays, it is apparent that the WHO AMH reference reagent is not being recognized in a similar manner to serum samples in all methods. The reason for the dissimilar responses between serum and WHO AMH reference reagent in these assays is not apparent (8). Clearly, the selection of kit reference preparation used in these methods is important, but other explanations regarding the different methodologies can be considered. From a global perspective one should not be too surprised by these results. AMH is a large complex glycoprotein which is found in the circulation in both precursor and processed forms (14, 15, 32). Little is known of the various heterogeneous AMH forms found in serum and it is unclear to what extent these forms are comparable with the purified recombinant preparation used as the reference reagent. Recently, AMH isoforms have been identified in human follicular fluid and granulosa cell extracts which do not match recognized consensus forms, suggesting that additional, as yet unknown, processing occurs (59). Additionally, the choice of antibodies used in the respective assays is also critical. Immunoassays of this sort are comparative assays where the adage 'like *vs*. like' strictly applies. For a serum

TABLE 2 | Challenges in creating a valid International reference preparation.

Considerations in the preparation of an International Standard	Challenge	Desired Outcome
Identifying a common reference preparation suitable in multiple diagnostic assays with differing specificities	Many circulating forms of AMH exist in the circulation. Although assays report that they detect and quantify the same target, they often have different specificities for those different circulating forms and so the preparation of a standard that is suitable for all assays becomes very challenging	The preparation of a standard that contains a "representative" mixture of all circulating forms may not be sufficient. It may be more appropriate to prepare separate standards for each circulating form. However, this may not have wide acceptance with all end users.
Choice of reference material e.g. plasma/serum-based, synthetic or recombinant i) effect on commutability; ii) sample volume and concentration	Concept of "like versus like" (reference material should behave in the same way as the samples being analysed) is especially relevant to the commutability of a reference material with clinical samples An International Standard is usually prepared as a large batch of vials or ampoules to be available for >10 years to prevent the need for end-users to regularly recalibrate their assays. The preparation of such a reference standard may require the pooling of patient samples to obtain sufficient material.	Since the general principle is that of "like versus like", often a standard is made using plasma or serum. Pooled plasma or serum with recombinant protein may be appropriate, but the requirement remains for the behavior of the standard to be the same as that of the test samples and that the standard is commutable with clinical samples across all assays. The pooling of patient samples or purification of the analyte from its native matrix or the substitution of the native analyte with a non-native version (e.g. recombinant) are manipulations that can change the nature of the standard to render it no longer commutable with patient samples. This is evaluated in a multi-method international collaborative study
Long term stability of reference material – effect on commutability	International Standards are expected to be stable for >10 years to prevent the need for regular recalibration exercises which can be expensive and problematic for end users. In addition, these materials must be shipped globally.	For these scientific and logistical reasons, the material needs to be formulated with specific stabilizing excipients and is usually lyophilized and potentially further altered in comparison to the native specimen matrix. The effects of these manipulations on the commutability of International Standards must be evaluated in the collaborative study. It may be possible to prepare a standard using unadulterated frozen material but the challenges associated with the long term stability and stability-on-shipping often prevent this approach.

The reader is referred to the WHO document 'Recommendations for the preparation, characterization and establishment of international and other biological reference standards' (60) for a fuller description.

assay, the most appropriate reference preparation should be serum-based, reflecting the samples under investigation, and yet the matrix used in the 16/190 preparation was bovine casein-based instead of human serum-based. The question of using a serum pool as a reference preparation was discussed by Ferguson and colleagues (8) but was not progressed due to problems of availability, standardization and continuity of a pooled serum standard supply. In contrast, the WHO AMH reference reagent satisfies many of the requirements expected of an international reference preparation and is the first widely available, stable, lyophilized preparation of AMH that can be used for harmonization of the current clinically relevant immunoassays. Its introduction should lead to greater consistency between the different kit assays. However, although the WHO reference reagent is likely to be commutable in a proportion of AMH assays, commutability with clinical samples has been demonstrated only in some but not all assays. As such, the reference reagent may not effectively harmonize the results for clinical samples in all assays, and because of this, has not been established as a WHO International Standard. Instead, its status as a WHO reference reagent represents its intended use as a common material with which manufacturers can investigate assay performance characteristics. This is critical as a first step in the continuum toward eventual AMH assay harmonization and will likely pave the way for a second generation of reference material(s) with which a more universal demonstration of commutability with clinical samples will be possible.

DISCUSSION

AMH immunoassays are now widely available for assessing ovarian reserve and have application in a number of reproductive conditions where the size of the ovarian reserve is clinically important. Immunoassays are now available both in automated and manual formats with the automated platforms showing superior assay characteristics. In addition, new sensitive immunoassays are now available for situations where AMH serum concentrations are low, as seen in young women following chemotherapy and in women approaching menopause.

At recent count there are AMH kits provided by more than 14 manufacturers, most with their own AMH reference preparations which to date have not been calibrated against a common (international) reference preparation. The WHO AMH Study (8) was an attempt to establish such an appropriate reference preparation to aid in the harmonization of assay results. One of the primary goals in the harmonization of immunoassays is that, for a given analyte, the same numeric result should be obtained for a clinical sample irrespective of the assay method used to derive that result. This facilitates the

derivation and effective use of clinical practice guidelines and supports evidence based medicine. A lack of harmonization can lead to different methods providing divergent results for the same clinical sample and clinicians and other healthcare professionals, who are often unaware of these differences, may wrongly classify a patient's health status. Central to improving agreement between the results of different assay methods is the traceability of calibration to reference preparations and there is now acceptance that these reference preparations should be commutable, i.e. they should behave in the same way as the native analyte itself. The mathematical definition of commutability in effect states that for two samples (e.g. test and reference), the ratio of the results from the samples will be the same for each assay method. Based on the lack of commutability of the WHO AMH reference reagent with serum samples in some assay methods, this preparation cannot be considered as a suitable universal immunoassay reference preparation, although it will play an intermediate role while a second generation preparation is identified. Identification of that preparation will require knowledge of serum forms of AMH so that a suitable compatible reference preparation can be identified. The current challenges of creating an international reference preparation are summarized in **Table 2**.

AUTHOR CONTRIBUTIONS

HL and WL conceived the idea of this article. HL and DR conducted literature search and wrote the manuscript, with critical input from CB and WL. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.691432/full#supplementary-material

Supplementary Figure 1 | Multiple forms of AMH generated by post-translation cleavage. The amino acid sequence is numbered from the N-terminus of the pre-proAMH in this figure. The proAMH molecule itself contains 230 amino acid residues.

Supplementary Figure 2 | Correlation between the Beckman-Coulter Gen II assay (Gen II), Access AMH assay (Access), and Elecsys AMH Immunoassay (Roche) for determination of AMH (n=94). The upper panels represent the Passing and Bablok regression plots whereas the lower panels represent the Bland-Altman plots. (Reproduced with permission from Li et al., 2016) (50).

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MINI REVIEW
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Ethnicity/Race and Age-Specific Variations of Serum AMH in Women —A Review

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Purpose of Review: In this review, we summarize ethnic/race- and age-related variation in AMH and discuss the underpinnings behind these differences.

Recent findings: Anti-mullerian hormone (AMH) has become a widely used method of ovarian reserve testing over the last 15 years. Numerous studies have shown substantial ethnic/race and age-related differences. When compared to age-matched Caucasian women, AMH levels tend to be lower in black and Hispanic women. Chinese women tend to have significantly greater AMH levels prior to age 25 than Caucasian women. When considering subpopulations within ethnicities, at least one study noted lower AMH levels among Maya women compared to other Hispanic women. Age exhibits a positive trend with AMH up until at least 25 years of age with a consistent decline after 34 years of age extending to menopause.

Summary: AMH levels are highly variable among ethnicities and race with higher age-matched levels typically seen in Caucasian women. Age does not exhibit a consistent linear relationship with AMH, but a consistent decline is seen starting in the third decade of life and proceeding to menopause.

Keywords: ovarian reserve, age, race, ethnicity, AMH, age-specific AMH levels

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INTRODUCTION

Anti-Müllerian hormone (AMH) as a marker of ovarian reserve is an essential aspect of infertility testing. This hormone which is also known as Müllerian inhibitory substance/factor (MIS/MIF) was first discovered in 1947. Since 2002, the role of AMH/MIS has expanded from its influence on Müllerian ducts to a method of gauging a woman's ovarian reserve (1).

AMH is part of the Transforming Growth Factor-beta (TGF-β) group of ovarian growth factor ligands. This family includes inhibins, bone morphogenic proteins (BMPs), activins, and growth and differentiation factors (GDFs). The 2750 bp gene for AMH is on the short arm of chromosome 19 which produces a 140kDa homodimer glycoprotein (2, 3). In-utero, AMH leads to the regression of the Müllerian ducts (4). In a male fetus, the SRY region of the Y-chromosome is expressed at approximately 8 weeks of gestation which then leads to AMH production in Sertoli cells. Once it has reached the Müllerian ducts, AMH causes the apoptosis of cells in these structures thereby leading to their regression. Due to the lack of the SRY region in female embryos, the Müllerian ducts

continue to develop into the uterus, fallopian tubes, and upper 2/3 of the vagina (5). Nonetheless, AMH is produced within females and is exclusively made by the granulosa cells within pre-antral and antral follicles (6) and was discovered to be present in women's pre-ovulatory follicles in 1993 (7).

Numerous unique properties have made serum AMH a mainstay of assessing a patient's ovarian reserve. In particular, AMH as a measure of ovarian reserve is more reliable due to its reduced variation within each menstrual cycle and reduced interobserver variability compared to antral follicle count (AFC) and follicle stimulating hormone (FSH) levels (7–9). Additionally, AMH demonstrates minimal cycle-to-cycle variability in comparison to AFC and FSH levels (10, 11).

Given this greater consistency, AMH has become a widely used tool to assess ovarian reserve (8, 12). Furthermore, it has been studied as a tool to guide and assess the potential oocyte yield prior to ovulation induction for assisted reproductive technology (ART) cycles. The first study to demonstrate a reliable correlation between AMH and egg yield showed that AMH levels were 2.5 times higher in patients whose ART cycles yielded 11 or more oocytes compared to those that yielded 6 or less oocytes. This correlation between ART success and AMH was extended to live birth rates. A retrospective study looking at 1230 in-vitro fertilization–intracytoplasmic sperm injection (IVF-ICSI) cycle outcomes indicated that the likelihood of live-birth increased in a log-linear fashion for an of AMH 2.94 and greater (13). Numerous additional studies have confirmed the correlation between higher AMH levels and superior ART cycle outcomes (14–16).

Ethnicity/race and age can have a substantial impact on ovarian reserve and thus, oocyte yield during ART cycles (17–19). In this review, we intend to address how AMH may vary by ethnicity/race and varies according to age.

METHODS

A search was performed in Pubmed, The Cochrane Library, and Ovid-Medline. Phrases used in the search were suited for each individual database and included "AMH AND Caucasian quality," "AMH AND African-American," "AMH AND black," "AMH AND asian," "AMH AND race," "AMH AND ethnicity," "AMH AND age," "AMH AND adolescents," "AMH AND menopause," "Müllerian inhibitory substance/factor" AND Caucasian," "Müllerian inhibitory substance/factor AND African-American," "Müllerian inhibitory substance/factor AND black," "Müllerian inhibitory substance/factor AND Asian," "Müllerian inhibitory substance/factor AND race," "Müllerian inhibitory substance/factor AND ethnicity," "Müllerian inhibitory substance/factor AND age," "Müllerian inhibitory substance/factor AND adolescents," and "Müllerian inhibitory substance/factor AND menopause. Our search period spanned from 1946-2020. 3352 articles were found. These articles were then assessed for relevance and quality. Only studies published in English were included. Thirty-three of these studies were included as part of this review. A manual review of the references in each of the cited sources was performed to ensure that any relevant resource was not excluded.

The primary outcome of this review was to determine if AMH levels were correlated at any age range and were associated with any ethnic/racial group. Articles were selected as relevant if they were: 1) prospective studies, retrospective studies or meta-analyses involving females who underwent AMH assessment between birth until menopause. Studies were excluded if they were 1) case reports, non-systematic reviews, abstracts, expert opinion articles, 2) did not include an analysis of patients that had an AMH drawn or only analyzed patients that has other markers of ovarian reserve such as AFC or basal FSH.

Ethnicity/Racial Differences

As described in the following sections, ethnicity/race has been associated with substantial variations in AMH levels. Ethnicity/race has typically been determined *via* patient self - reporting. However, genetic markers of ethnicity/race, also known as ancestry informative markers (AIMs), have been seen as a more objective method of ascertaining a patient's ethnicity. Olcha et al. looked at various ovarian reserve markers across various ethnicities based upon genetic ancestry *via* AIMs. This group showed that when controlling for age and body mass index (BMI), there is no variation in AMH based upon genetic markers of ethnicity (20). Determining ancestry *via* AIMs does have limitations which include the limited reference dataset of single-nucleotide polymorphisms that are used to identify genetic ethnicity (21). Consequentially, the use of AIMs may artificially contract the cohort of patients assigned to any one ethnicity. Therefore, we will assess the available literature on variations in AMH levels based upon patient-reported ethnicity.

Caucasian

Caucasian women are often the reference standard when assessing ethnic differences for a variety of parameters in infertility (22). Hence, we will first assess this ethnic/racial group. The first study looking at ethnic differences in AMH levels showed higher levels in Caucasian women compared to Black and Hispanic women after controlling for age, BMI, smoking, and HIV status (23). The second and one of the largest comparative assessments of ovarian reserve values among ethnicities was performed by Bliel et al. In this cross-sectional study they assessed AMH levels in 947 women, of which 277 were white, 237 were African-American, 220 were Latina, and 213 were Chinese. Compared to all other ethnic groups, average AMH levels were consistently greater in white women, until age 35 (24). Substantial research has looked to variations in AMH levels in Caucasian women according to the presence of polycystic ovary syndrome (PCOS). Moy et al. performed a retrospective analysis looking at factors that affect AMH among numerous racial/ethnic groups. While age did correlate negatively with AMH among all ethnic groups, the prevalence of polycystic ovary syndrome, smoking, and elevated BMI correlated negatively only in Caucasian women (25). Of note, no distinction was made between infertility status of the women in any of the aforementioned studies.

African-American/Black

Numerous studies have assessed AMH levels according to pre- or post-menopausal status. Based upon the study by Seifer et al., AMH was 25.2% lower in Black women compared to Caucasian women which was independent of age, BMI, smoking, and HIV status (23). In the study by Bleil et al., this group showed that their cohort of 237 Black and 213 Chinese women of younger and middle ages exhibited lower AMH levels compared to 227 white women. However, AMH levels in black women were higher than Latina and Chinese women of an older age (24). Marsh et al. looked at factors which lead to the variation in AMH levels in 1,654 African-American Women (AAW). Median AMH for AAW was 3.18 ng/ml and, in their age-adjusted model, they showed that BMI, use of hormonal contraceptives, and history of a thyroid disorder were negatively correlated with AMH levels. Furthermore, a history of abnormal bleeding during menses and oligomenorrhea was associated with higher AMH levels (26). Just as in Caucasian women, obesity, especially if patients were obese at 18 years of age had a significant negative correlation with AMH levels (27). This variation in AMH may be one contributing factor among several underlying the consistently lower live birth rates from assisted reproductive technologies observed in black women in the US (28). Looking at post-menopausal women, a cross-sectional study which included 671 women without a history of malignancy showed that race was not significantly associated with AMH levels (29). Furthermore, the study by Bleil et al. showed in a cohort of 947 women that AMH levels varied in a more consistent fashion across ethnicities among older women, especially when approaching the age of perimenopause (24). As with the studies in assessing AMH in Caucasian women, the studies assessing AMH levels in Black women did not distinguish between fertile and infertile populations.

Hispanic

As with black women, substantial differences have been noted in AMH levels with Hispanic women compared to other ethnic groups. Although the difference was not statistically significant, Seifer et al. showed a 24.6% lower AMH level in Hispanic women than in Caucasian women (22). Bleil et al. showed that, across all ages, AMH was lower in a cohort of 220 Hispanic women compared to the 227 Caucasian women included in the study (24). Even among Hispanic women, substantial variations in AMH levels exist. In one study looking at women of Maya heritage in Mexico, these women were over five times more likely to have undetectable AMH levels compared to non-Maya women (30). Further research into regional or ancestral differences, especially throughout the Caribbean, Central, and South America may yield additional nuances in ovarian reserve levels in this population.

Asian Populations

Examining populations throughout Asia, there are two distinct trends seen. In a Chinese study looking at over 6,700 women from birth to the post-menopausal age, the authors noted a peak in AMH at age 18 with a consistent decline up until age 50 (31). When compared to Caucasian women, Nelson et al. recently noted that AMH levels were typically higher in Chinese women up until 25 years of age and then AMH levels tended to be less than Caucasian women after age 25 (32).

This is not the case in the South Asian population. Bhide looked at AMH variations in 865 women at a single fertility clinic and showed that despite higher AMH levels seen in South Asian patients compared to Caucasians, this difference disappeared in their multivariable analysis (33). These results are consistent with data from ART cycles which showed no significant difference between 236 Indian women and 236 Spanish women as far as AFC. AMH was slightly lower among Spanish women, but this was likely a factor of their older age (34). While differences may be present between younger Chinese and Caucasian women, this disparity disappears in older women and especially within other populations in Asia.

Genetic and Environmental Underpinnings Behind Ethnic/Racial Differences

Several groups have looked relationships between AMH levels and genetic variations among different ethnicities. Schuh-Huerta et al. looked at genetic variants and AMH levels in 232 Caucasian women and 200 African-American women. They showed two nominal genetic variants in the Jumonji, AT-rich interactive domain 2 (JARID2) gene and AMH levels in both ethnic groups (35). A separate group looked at fragile-X mental retardation (FMR1) and found that AMH level was associated with the number of repeats in the FMR1 gene ($p < 0.001$) (36). This group correlated FMR1 variants with ethnicity and found substantial differences with Caucasians having the highest prevalence of abnormal alleles in this gene (37). BRCA1 has also been associated with lower AMH levels and BRCA1 is known to be more prevalent among non-Hispanic whites, and African-Americans (38, 39). Currently, it is unclear if any of these genetic factors lead to lower AMH levels primarily as a result of ovarian aging or rather as a result of altered AMH expression/secretion. Interestingly, at least one paper noted a paradoxical increase in AMH mRNA and protein in cumulus cells and follicular fluid as patient age increased (40).

Environmental factors may certainly also influence the ethnic variation of AMH. Numerous studies have shown a negative association between obesity, smoking and AMH levels (41–43). A multicenter study looking at the effect of BMI on the AMH levels of patients with PCOS and ovulatory controls, showed that BMI exhibited an inverse relationship with AMH regardless of patients age, race, smoking status and site in their regression analysis (44). This was seen in both cohorts. Plante et al. in their single-center cohort study looking at the effect of self-reported smoking history on AMH showed that current, but not past smokers have a 44% lower AMH level. Of note, they did not notice a dose-dependent response (45). Furthermore, these factors are known to be more prevalent in the African-American and Hispanic populations (46). Additional nutritional factors may include vitamin D deficiency which is more prevalent among women of color and may in part contribute to lower serum AMH levels in these patient populations (46, 47). However, additional mechanistic studies of these factors are needed to confirm these environmental/

nutritional effects as direct drivers behind ethnic/racial disparities in AMH levels.

Age-Related Differences

From Infancy to Adolescence

AMH exhibits a progressive trend in the early stages of life. A Danish study from 2010 in examined AMH levels in 926 healthy females from birth to adulthood. They noted that AMH was undetectable in 54% of cord blood samples (i.e. from infants). AMH then increased from birth to 3 months up to 15 pmol/liter (6.6 ng/ml). Then from 8-25 years, AMH levels remained stable with the average level being 19.9 pmol/liter (8.8 ng/ml) (48). This is consistent with a subsequent study which showed no difference in AMH levels in the American female population between 10–21 years of age (49).

Adulthood

Concerning AMH levels in older women, Lie Fong et al. (45) expanded on the work done by the Hagen et al. (45) by looking at AMH levels from birth to older adult ages. They noted that AMH climbed to its maximum by 15.8 years of age and then remained stable until 25 years of age at which point it started a progressive decline to menopause. With older women, AMH was negatively correlated with age (50). This correlation was modeled in a linear fashion; however both Lie Fong et al. and La Marca et al. showed that the relationship is better fitted with a polynomial function (50, 51). Overall, the relationship between AMH and age cannot be considered linear throughout all age ranges.

When considering ovarian reserve and fertility, it is essential to assess when the decline in AMH becomes more precipitous. Wiweko et al. (49) did a retrospective study that looked at the relationship between various markers of ovarian reserve including FSH, AFC, and AMH. Serum AMH was shown to decline after 34 years of age. Bozkurt et al. (50) also noted an age-related decline in AMH in both the fertile and infertile patients they studied. While they did not note an age at which the decline in AMH accelerated, patients 35 and older maintained a consistent age-related decline in AMH in both the fertile and infertile groups. Concerning the magnitude of this decline in the infertile population, the rate of decline of the median AMH was noted to decrease in a retrospective review of over 17,000 infertile women from 0.2 to 0.1 ng/ml per year after age 35 (52). Please see **Figure 1**. A subsequent prospective study comparing infertile women younger than 40 and fertile controls of the same age range noted an approximately 6% decrease in AMH per year (53). These findings supplement the findings in the infertile population from Seifer et al. (52). Thus, there are age-specific values that are clinically useful to keep in mind when using AMH in the infertile population to determine the status of a woman's ovarian reserve. Of note, a retrospective study of healthy, reproductive age women showed that even at younger ages, concerningly low AMHs were noted. This suggests that even young women do have some risk for low ovarian reserve and that caution is needed when counseling patients on fertility planning (54).

Clinical Significance

Our knowledge of the ethnicity- and age-related variation in AMH allows for giving proper context to patients. While patients of various ethnicities may exhibit lower levels of AMH, patients can be reassured that, the general trend of AMH with age appears to be consistent among ethnicities. Furthermore, the age-based variation is an effective tool to gauge possible oocyte yield for patients undergoing treatment with assisted reproductive

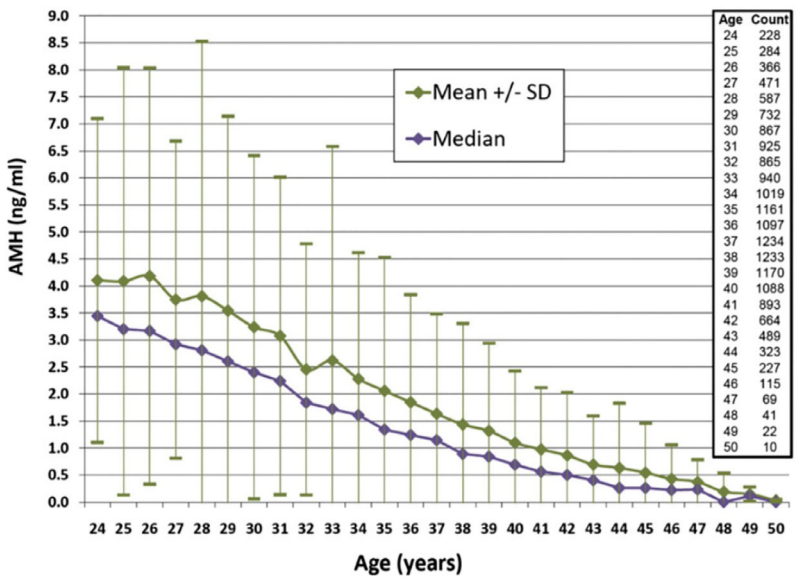

FIGURE 1 | AMH levels according to age for women 24–50 years of age. Median values are shown with standard deviations. Reproduced with permission from Seifer et al. Age-specific serum anti-Mullerian hormone values for 17,120 women presenting to fertility centers within the United States. Fertil Steril. 2011; 95 (2): 747-50.

technologies (55). The age-dependent decline in AMH can also assist in gauging response in women undergoing fertility preservation (55).

CONCLUSIONS

AMH, as a mainstay of ovarian reserve testing, shows widespread ethnicity/race- and age-based variation. Variations according to ethnicity/race may be dependent on additional factors such as BMI, PCOS, socioeconomic status, environmental/nutritional factors such as vitamin D status among other aspects. However, the effect of age on AMH appears to be consistent among various ethnicities/races and thus, age-specific AMH values provide clinical context particularly in the infertile woman. Age is a strong common denominator that influences the reproductive life of women regardless of race or origin. Future research may shed light on how to mitigate the effects of ethnicity/race and age upon AMH as a reflection of ovarian reserve. This future work could potentially have a favorable impact on ART and fertility/family planning in general.

AUTHOR CONTRIBUTIONS

AK and DS contributed equally to the article search, analysis, review, and compilation of relevant articles for this review. All authors contributed to the article and approved the submitted version.

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REVIEW
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What Does AMH Tell Us in Pediatric Disorders of Sex Development?

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Disorders of sex development (DSD) are conditions where genetic, gonadal, and/or internal/external genital sexes are discordant. In many cases, serum testosterone determination is insufficient for the differential diagnosis. Anti-Müllerian hormone (AMH), a glycoprotein hormone produced in large amounts by immature testicular Sertoli cells, may be an extremely helpful parameter. In undervirilized 46,XY DSD, AMH is low in gonadal dysgenesis while it is normal or high in androgen insensitivity and androgen synthesis defects. Virilization of a 46,XX newborn indicates androgen action during fetal development, either from testicular tissue or from the adrenals or placenta. Recognizing congenital adrenal hyperplasia is usually quite easy, but other conditions may be more difficult to identify. In 46,XX newborns, serum AMH measurement can easily detect the existence of testicular tissue, leading to the diagnosis of ovotesticular DSD. In sex chromosomal DSD, where the gonads are more or less dysgenetic, AMH levels are indicative of the amount of functioning testicular tissue. Finally, in boys with a persistent Müllerian duct syndrome, undetectable or very low serum AMH suggests a mutation of the AMH gene, whereas normal AMH levels orient toward a mutation of the AMH receptor.

Keywords: testis, ovary, Turner syndrome, Klinefelter syndrome, persistent Müllerian duct syndrome, gonadal dysgenesis, Sertoli cell, Leydig cell

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INTRODUCTION

DSD can be defined as conditions where genetic, gonadal, and/or internal/external genital sex are discordant. The most common cause of DSD, congenital adrenal hyperplasia (CAH), is easily recognized, but other conditions may be more difficult to diagnose. Anti-Müllerian hormone (AMH), produced in large amounts exclusively by the fetal and prepubertal testis, is an important parameter for differential diagnosis, particularly in children. AMH plays a key role in male sex differentiation.

DIFFERENTIATION OF THE GENITAL TRACT

Initially, the internal reproductive tract is identical in XX and XY embryos. Mesonephric (Wolffian) ducts form in the intermediate mesoderm during the 4th week and elongate caudally in direction of the urogenital sinus (1). During the 5th week, coelomic cells specified to become Müllerian cells form a cleft between the gonadal and mesonephric ridges, laterally to the Wolffian ducts. Then, these cells invaginate caudally until they reach the Wolffian duct, a step requiring the expression of WNT4 by the mesonephric mesenchyme (2). The Müllerian ducts grow toward the urogenital

sinus, crossing the Wolffian ducts ventrally, thus finally lying medially and fusing to give rise to the uterovaginal canal in the midline (3). Elongation of the Müllerian duct is regulated by WNT9B secreted by the Wolffian duct epithelium (4) and requires physical contact with the latter (3).

The subsequent fate of the Müllerian duct differs markedly according to sex. In the normal male, its cranial end shows signs of impending regression even before the Müllerian duct reaches the urogenital sinus, coinciding with the beginning of secretion of AMH by Sertoli cells. The Müllerian duct morphologically resembles an epithelial tube but expresses mesenchymal cell markers. These mesoepithelial characteristics persist during regression while, at the same age, the female Müllerian duct becomes exclusively epithelial, heralding the end of the window of sensitivity to AMH (5). Müllerian regression is characterized by loss of the epithelial basement membrane and by apoptosis, progressing toward the urogenital sinus. In the human fetus at 9 weeks, Müllerian ducts have nearly totally disappeared.

Leydig cells, under the effect of placental human chorionic gonadotropin (hCG), produce testosterone, which acts directly on the Wolffian ducts promoting their differentiation into epididymides, vasa deferentia, and seminal vesicles by the 12th week. Male differentiation of the genitalia, including fusion of the labioscrotal folds to form the scrotum, closure of the urethral folds, and positioning of the meatus at the tip of the phallus, is completed by the end of the first trimester of fetal life. The genital tubercle forms the corpora cavernosa and corpus spongiosum of the penis. In the second half of gestation, fetal pituitary LH takes over the regulation of testosterone production, which drives the increase in penile size and—together with the Leydig cell factor insulin-like 3 (INSL3)—the descent of the testes to scrotal position.

In the absence of testicular hormones, whether ovaries are present or not and irrespective of karyotype, differentiation of the genital tract follows the female pathway. In the absence of AMH, the Müllerian ducts form the Fallopian tube, the uterus, and the upper part of the vagina. They become resistant to AMH when they lose their mesenchymal markers to become purely epithelial (5). In the absence of androgen action, Wolffian ducts regress through an active process induced by COUP-TF2 (6). The prostate does not differentiate, and the vagina opens separately from the urethra on the surface of the perineum. The urethral folds do not fuse and give rise to the labia minora; the labioscrotal swellings also remain separated to form the labia majora. The genital tubercle does not grow and forms the clitoris. For a detailed description of sex differentiation, see ref. (7).

AMH, A MEMBER OF THE TGF-BETA FAMILY

AMH, a member of the transforming growth factor beta (TGFβ) family (8) secreted by Sertoli cells immediately after testicular differentiation, is responsible for the regression of Müllerian ducts in the male fetus. Like other members of the TGFβ family, AMH is translated as a dimeric precursor protein comprising two polypeptide chains, each containing a large N-terminal pro-region and a much smaller C-terminal mature domain homologous to those of the other members of the TGFβ family. Proteolytic cleavage at arginine 451 yields 110-kDa N-terminal and 25-kDa C-terminal dimers which remain associated in a bioactive non-covalent complex (9). The human AMH gene (8), only 2.8 kb long and located on chromosome 19p13.3 (10), contains five exons; the 3′ end of the fifth exon encodes the bioactive C-terminal domain. GATA, SF1, and SOX9 binding sites present within 418 bp of the translation start site activate AMH expression; sites further upstream are required for normal regulation (11).

Like other members of the TGFβ family, AMH signals through two membrane-bound serine/threonine kinase receptors and several cytoplasmic R-SMAD effectors. The primary receptor, *AMHR2* gene contains 11 exons spread over 8 kbp and maps to chromosome 12q13.13 (12). After binding of AMHR2 to its specific ligand AMH, the complex recruits a type I receptor, either the BMP receptors BMPR1A, aka ALK3, or BMPR1B, aka ALK6, or the activin receptor ACVR1, aka ALK2, resulting in the phosphorylation of SMADS 1, 5, or 8. The type I receptor and SMAD repertoire is shared with the bone morphogenetic protein family, AMH's closest relatives within the TGFβ family (7).

Tight regulation of AMH transcription is crucial for regression of Müllerian ducts since they are able to respond to AMH only within a very narrow time window (13). Surprisingly, however, production does not cease once Müllerian ducts have disappeared; it continues up to puberty. Initially, transcription requires the cooperation of various transcription factors, SOX9, SF1, GATA4, and WT1, to name only a few [reviewed in (14)]. Later in fetal life and after birth, testicular AMH production is increased by FSH signaling through its seven-transmembrane receptor and the pathway involving the Gsα subunit (15), cyclic AMP, protein kinase A, and transcription factors SOX9, SF1, AP2, and NFκB (11) (**Figure 1**). Transcription is downregulated by androgens (16). Testosterone acts through the androgen receptor and requires intact binding sites for SF1 on the AMH promoter (17) (**Figure 2**).

WHY MEASURE AMH IN DSD?

Müllerian ducts have completely disappeared in the male 10 weeks after conception, but testes continue to churn out high amounts of AMH throughout childhood, when basal testosterone and gonadotropin levels have little clinical use. It is precisely the fact that AMH continues to be secreted at high levels by Sertoli cells during infancy and childhood, when it has no longer a physiological action on Müllerian ducts, which makes AMH such an appealing biomarker for pediatric endocrinologists, not to mention that prior gonadotropin stimulation is not required. In theory, to determine whether AMH has been secreted or not in a DSD patient, a look at Müllerian derivatives by laparoscopy or sonography should suffice. However, laparoscopy is invasive, and sonography is not always reliable in newborns. Measurement of AMH concentration in serum by ELISA has been available since 1990 and is now offered by multiple companies (please refer to another article devoted to AMH assays in the same

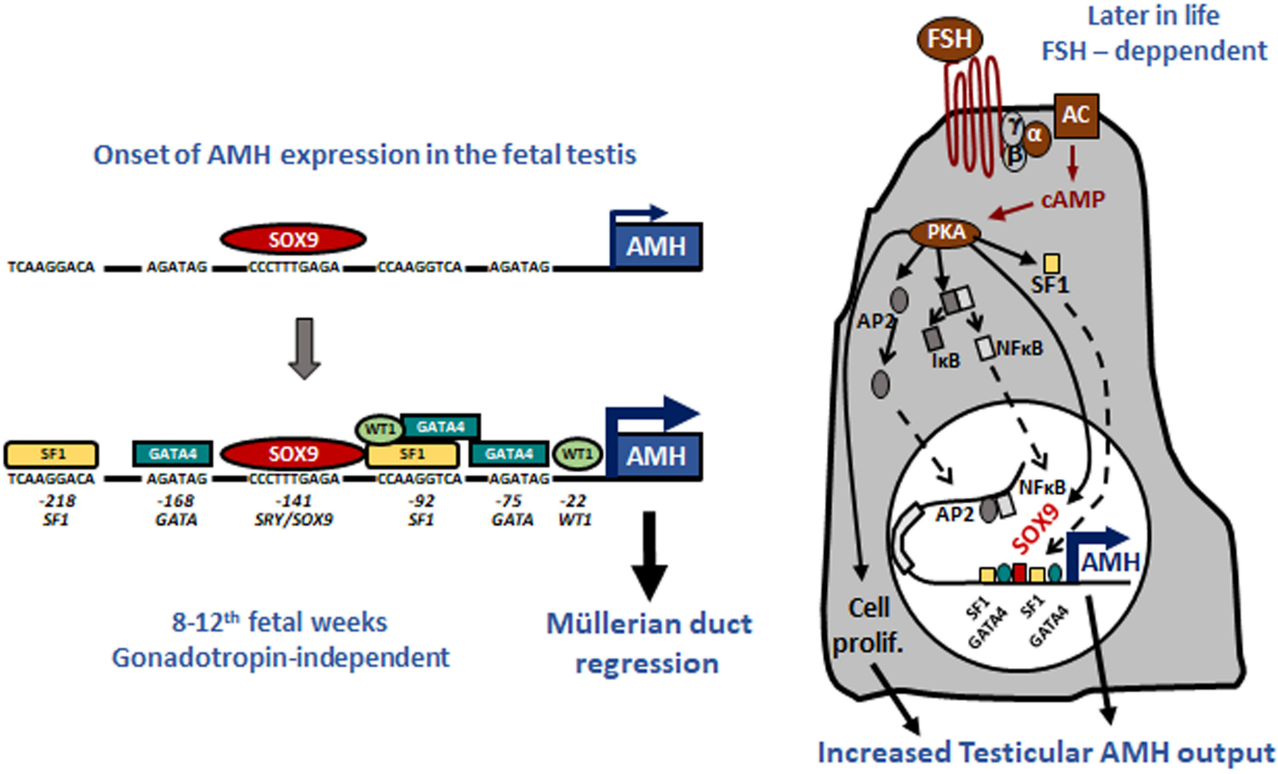

FIGURE 1 | Upregulation of AMH production in testicular Sertoli cells. Left: onset of *AMH* expression in early fetal life is independent of gonadotropins and depends on transcription factors SOX9, which triggers *AMH* expression, and SF1, GATA4, and WT1, which further increase *AMH* transcription by binding to specific response elements on the proximal *AMH* promoter. Right: increase of testicular AMH production in response to FSH, involving the FSH receptor-Gsα protein-adenylate cyclase (AC)-cyclic AMP (cAMP) pathway, which activates protein kinase A (PKA)-mediated induction of SOX9, SF1, NFκB, and AP2. These factors bind to their specific response elements on the *AMH* promoter. Reproduced with permission from ref. (11). Copyright© 2011 the American Physiological Society and © 2020 MDText.com, Inc.

series). However, the information provided by imaging and AMH measurement is not similar. The state of the Müllerian derivatives reflects the effect of AMH secreted very early in fetal life, while serum AMH reflects the amount secreted at the time blood is drawn, and the two do not necessarily coincide (18).

The concentration of AMH, one of the first proteins produced by the fetal testis, is high in serum during fetal life though not detectable in amniotic fluid (19). Although it declines transiently at birth, it remains distinctly higher in males than in females and increases again during the 1st month (20) to reach its peak in the 2nd year of life (21, 22). Circulating levels remain high during childhood but fall at the onset of puberty, downregulated by the rising intratesticular concentration of testosterone. The normal AMH serum concentration in developing boys is shown in **Figure 3**. Interestingly, during the fetal period and early infancy, Sertoli cells do not express the androgen receptor and are thus insensitive to the high levels of testosterone of minipuberty (23–25) and references therein.

What does AMH tell us in DSD? Serum concentrations clearly above female values indicate that testicular tissue is present. Testicular tissue virilizes the fetus through the combined action of two hormones, AMH and testosterone. If both are defective, global testicular dysgenesis is probably involved. If only one is deficient, a block in the synthesis or action of either testosterone or AMH is likely. Serum AMH is also a reliable biomarker of the balance between FSH and androgen action within the testis (26): high AMH indicates FSH stimulation and lack of androgen action as in androgen insensitivity whereas low AMH suggests a predominant androgen inhibiting action, as in precocious puberty. Furthermore, in prepubertal DSD patients, serum AMH measurement monitors Sertoli cell function, a very useful feature since testicular tissue tends to deteriorate over time in DSD.

In the ovary, low amounts of AMH are produced by the granulosa cells of primary and small antral follicles (27–29), starting from the 25th week of fetal life (30). At that time, Müllerian derivatives are no longer sensitive to AMH (5, 31). Serum AMH is 50-fold lower in girls than in boys at birth (20) and remains relatively stable from childhood through young adulthood (32).

THE 46,XY CHILD WITH DSD

According to the original DSD consensus statement (33) updated in 2016 (34), DSD are initially classified according to the

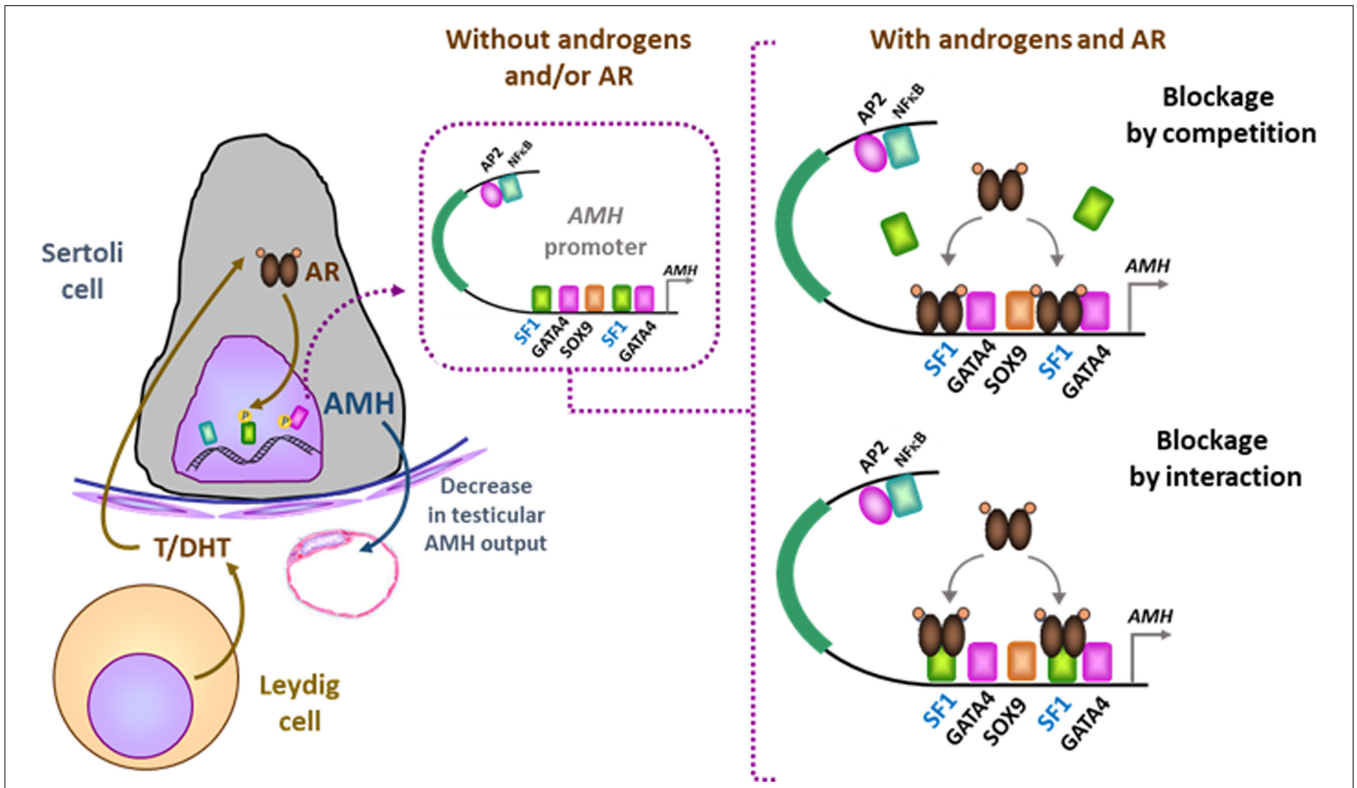

FIGURE 2 | Downregulation of AMH production in testicular Sertoli cells by androgens. In the absence of androgens (e.g., normal childhood and DSD with impaired androgen production) or androgen receptor (AR, e.g., normal fetal and neonatal periods and DSD due to androgen insensitivity syndromes), SF1 binds to its response elements on the *AMH* promoter and increases *AMH* transcription. When androgen action on the AR is effective in the Sertoli cell (e.g., at puberty), the ligand-activated AR could either block SF1 binding to the *AMH* promoter (blockage by competition) or interact with site-bound-SF1 and prevent it from exerting its stimulatory effect on *AMH* promoter (blockage by interaction). Modified with permission from (17). Copyright © The Author(s) 2018.

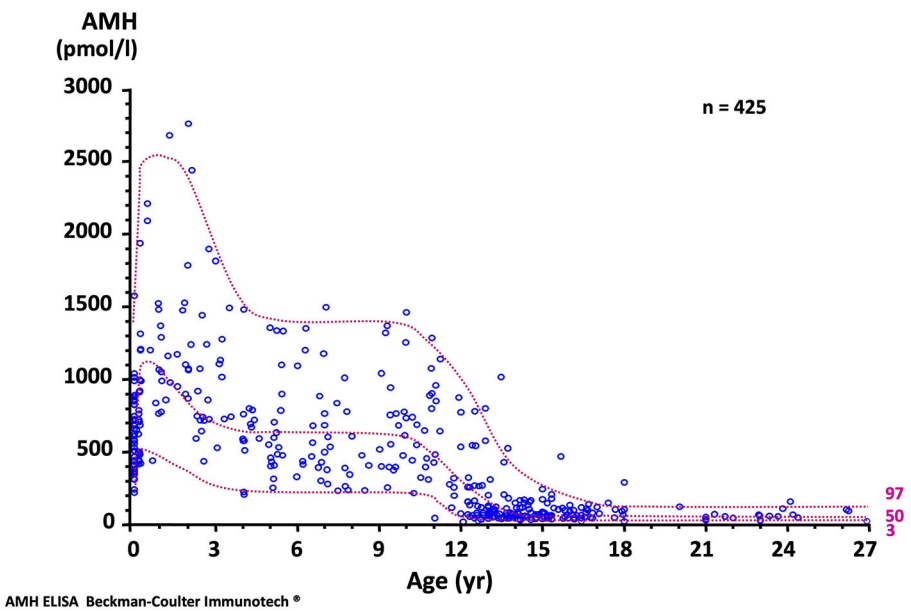

FIGURE 3 | Serum AMH levels in normal males. The dotted lines represent the 97, 50, and 3rd percentiles. To obtain serum AMH in ng/mL, divide by 7.14. Reproduced with permission from ref. (22). Copyright © The Author(s) 2011.

TABLE 1 | Classification of disorders of sex development (DSD) according to the karyotype and the underlying pathogenesis.

Affected process	46,XY	46,XX	Chromosomal
Gonadal differentiation	Complete (or Pure) gonadal dysgenesis Partial testicular dysgenesis	XX male Ovotesticular DSD	Asymmetric gonadal differentiation (or mixed gonadal dysgenesis) Ovotesticular DSD Klinefelter and Turner syndromes
Androgen production (isolated)	Leydig cell aplasia/hypoplasia Steroidogenic defects	Congenital adrenal hyperplasia Aromatase deficiency Exposure to maternal androgenic tumors or drugs	None
Androgen action (isolated)	CAIS / PAIS	None	None
AMH production or action (isolated)	PMDS	None	None

CAIS, Complete androgen insensitivity syndrome; PAIS, Partial androgen insensitivity syndrome; PMDS, persistent Müllerian duct syndrome.

patient's karyotype into 46,XY, 46,XX, and sex-chromosome DSD (**Table 1**). Incomplete or total lack of virilization of the external genitalia in 46,XY individuals may result from insufficient testosterone production by the gonads or defective androgen action at the target organ level. If the deficiency is complete, genitalia have a normal female appearance at birth (**Figure 4**), and the condition may go undiagnosed until puberty, unless a karyotype is performed for other reasons, before or after birth. Partial defects result in genital ambiguity leading to earlier medical intervention.

Combined AMH and Testosterone Insufficiency: A Hallmark of Gonadal Dysgenesis

Testosterone, a steroid responsible for genital virilization, and AMH, a glycoprotein member of the TGFβ family, are entirely different molecules with specific biosynthetic and signaling pathways. A defect in both implies that the entire testis is abnormal. In the most extreme scenario, genitalia are completely female and Müllerian derivatives; uterus and Fallopian tubes are normal, suggesting that testes have never existed or functioned in the first place (**Figure 4D**). Serum AMH is undetectable (35, 36) (i.e., even lower than in 46,XX girls). This condition, known as **pure (or complete) gonadal dysgenesis** or Swyer syndrome, is the complete form of early fetal-onset primary gonadal failure (37, 38) and is sometimes labeled "sex reversal" although gonads are represented by streaks, not ovaries.

Primary Gonadal Failure May Be Partial

The external genitalia are partially virilized, resulting in micropenis and hypospadias or clitoral hypertrophy and labial fusion in a female perspective. Ultrasound examination usually reveals the presence of more or less developed Müllerian derivatives (**Figure 4E**). The serum AMH concentration is lower than in boys of similar age. Testosterone response to hCG is also blunted, but this in itself does not rule out the possibility of an isolated defect in Leydig cell steroidogenesis or a mutation in the LH/CG receptor. However, in the latter conditions, AMH is produced normally, there are no Müllerian derivatives, and AMH concentration is normal for age and sex. A frequent variant of partial gonadal failure is represented by mixed or asymmetrical gonadal dysgenesis (39), characterized by the presence of a testis on one side and a streak gonad on the other (40). These patients often exhibit congenital malformations evocative of Turner syndrome, and their karyotype is typically 45,X/46,XY. Müllerian derivatives are often absent on the testicular side. Familial cases of complete or incomplete gonadal dysgenesis are frequent. Molecular defects affecting the testis determination pathway (41) are often detected. In contrast to autosomal recessive steroidogenic defects, gene haploinsufficiency is often sufficient to disrupt testicular differentiation.

All patients with XY gonadal dysgenesis are at risk for malignant germ cell tumors and should be closely monitored (42). In itself, presence of Müllerian remnants should alert the clinician to this possibility. Formerly, cancer risk was a decisive argument for choosing a female sex of rearing in poorly virilized patients with Müllerian organs, because the decision involved early bilateral gonadectomy. Today, some human rights activists consider that genitoplasty of infants should be outlawed [see (43–45)], with the result that dysgenetic testes may be left *in situ* until the child reaches prepuberty (46), a particularly dangerous age for the onset of germ cell malignancy (47).

Isolated Testosterone Insufficiency: Steroidogenesis Defect or Testosterone Insensitivity?

AMH is also helpful if an androgen synthesis or action defect is suspected in a patient with DSD. Indeed, ambiguous or female genitalia are indicative of androgen failure, but testosterone levels cannot distinguish between gonadal dysgenesis and specific Leydig cell disorders or androgen insensitivity during childhood. Serum AMH can orient the diagnosis: it is low or undetectable in gonadal dysgenesis, but normal or high in isolated Leydig cell disorders or androgen insensitivity.

Testosterone Synthesis Defects

The initial steps of steroidogenesis are shared by the adrenals and the gonads; thus, defects of testosterone synthesis are often

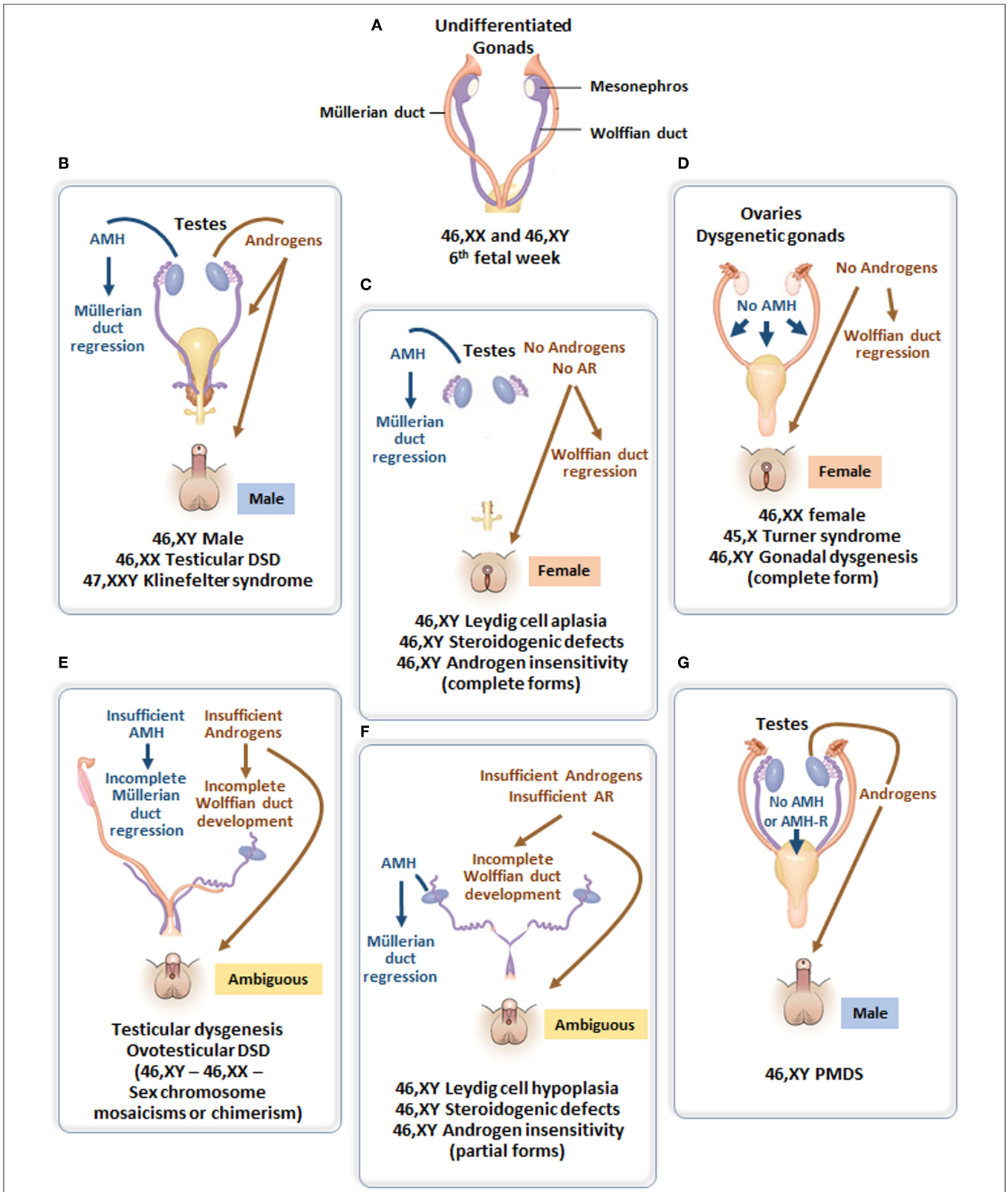

FIGURE 4 | (A) Undifferentiated stage of fetal sex development. **(B)** Male differentiation in normal 46,XY individuals and patients with 46,XX testicular DSD or Klinefelter syndrome. **(C)** Female external genitalia in patients with 46,XY DSD due to impaired androgen synthesis or action. **(D)** Female genital differentiation in normal 46,XX individuals and patients with dysgenetic DSD associated with 46,XX, 45,X or 46,XY genotypes. **(E)** Ambiguous external genitalia in patients with testicular or ovotesticular dysgenesis with different karyotypes. **(F)** Ambiguous external genitalia in patients with 46,XY DSD due to impaired androgen synthesis or action. **(G)** Male external genitalia and persistence of Müllerian ducts in 46,XY patients with *AMH* or *AMHR2* gene defects.

associated with rare forms of congenital adrenal hyperplasia (48) (**Figure 5**). Briefly, cholesterol is transferred to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) in response to LH or hCG and then converted to pregnenolone by the cytochrome P450 side chain cleavage (P450scc), an enzyme located at the inner mitochondrial membrane. 3ß-Hydroxysteroid dehydrogenase (3ß-HSD) and ultimately 17β-hydroxysteroid dehydrogenase (17ß-HSD) sequentially synthesize testosterone from the pregnenolone precursor. P450 oxido-reductase (POR) serves as an electron donor for all microsomal cytochrome P450 enzymes (50). Mutations in any of these enzymes or in the LH/CG receptor curtail hCG-stimulated testosterone production to levels observed in patients with gonadal dysgenesis (51). However, the level of serum AMH is normal or elevated and no Müllerian derivatives are detectable, allowing to make the distinction (**Figure 4C**). The identification of the defective enzyme requires the assay of steroid hormone precursors prior to Sanger sequencing of the suspect gene unless massive parallel sequencing targeted to DSD genes is chosen as a shortcut. All steroidogenic defects are transmitted as recessive autosomal traits, including those that affect testosterone metabolism.

Dihydrotestosterone Synthesis Defects

Testosterone can be converted to its more potent metabolite DHT by the enzyme 5α-reductase type 2 (52) expressed in androgen target organs such as fetal genital skin, male accessory sex glands, and prostate. Mutations in the SR25A2 gene are the most frequent cause (53, 54). Alternatively, DHT can be synthesized through the "backdoor" pathway without going through a testosterone intermediate (55). Defects in DHT production lead to very poor virilization of target organs, and most patients are considered girls at birth. Serum testosterone is unremarkable; the T/DHT ratio is high, after hCG stimulation if necessary. The AMH level is in the lower range of normal (56), indicating that testosterone does not need to be metabolized to DHT to regulate AMH production by the Sertoli cell. From a practical viewpoint, it is very important to distinguish 5α-reductase deficiency from other types of XY DSD because if raised as girls, patients often switch to a male sex at puberty, an unusual occurrence in androgen insensitivity.

Androgen insensitivity is the consequence of a mutation in the androgen receptor that is coded by a gene on the X chromosome; transmission is recessive sex-linked, affecting only males who lack a normal X chromosome. Again, the condition can be complete (CAIS), characterized by an external female phenotype or partial (PAIS), leading to ambiguous genitalia. In both instances, Müllerian ducts are normally regressed (**Figure 4C**). The level of serum AMH depends on the age of the patient. During the 1st year of life and at puberty, AMH levels are extremely high, reflecting the stimulation of AMH by FSH unimpeded by testosterone action (57). Serum testosterone is also elevated. Pubertal maturation is female in CAIS, except that menstruation cannot occur. In children, at present, molecular analysis of the androgen receptor is the diagnostic method of choice since testosterone and gonadotropin levels overlap for PAIS, 5α-reductase type 2 deficiency and steroidogenic defects.

In summary, in undervirilized XY DSD, low AMH is typical of gonadal dysgenesis (1 and 3 in **Figure 6**) while normal to high levels are observed in androgen insensitivity and androgen synthesis defects (2 and 4 in **Figure 6**). In most other forms of XY DSD linked to testosterone insufficiency, the AMH serum level is normal for age and diagnosis rests essentially on the assay of testosterone and its precursors as well as metabolites and gonadotropins. However, biochemical results are not always clear-cut, and today, molecular analysis is assuming an increasingly important role. Targeted or whole exome new-generation sequencing has significantly improved the proportion of diagnosed XY DSD cases (58, 59).

The Male Child With Impalpable Testes

At first glance, a newborn boy with impalpable testes is not a DSD candidate, unless his karyotype dictates otherwise. Once a 46,XY karyotype has been ascertained, the pediatrician must choose between two possibilities: bilateral cryptorchidism or anorchia. AMH is very helpful in this regard if its level is higher than the normal female value, testicular tissue must exist (60, 61) because the prepubertal testis is the only organ capable of secreting large amounts of AMH.

Cryptorchidism is frequently observed in newborns, particularly in preterm ones. To test for the presence of testicular tissue, basal testosterone assay and gonadotropin measurement may not be informative, depending on age. In contrast, an AMH level above female values establishes without a doubt that testicular tissue is present and is an indication for surgery if the testes do not descend spontaneously within the first 6 months of life. AMH is moderately decreased in cryptorchidism; even unilateral but still much higher than in females (62), an hCG test is not required.

In patients with impalpable testes, **anorchia**, although rare, is a possibility that should be considered. Since the external and internal phenotype is unequivocally male, it is obvious that testes were present at least up to month 4 of fetal life and disappeared after fetal sex differentiation was completed. Indeed, in some instances, testes were palpable at birth and vanished later. The condition is apparently due to degeneration subsequent to testicular torsion, and no genetic alterations were detected in 26 cases (63). AMH is not detectable in serum; however, ultrasound examination should be performed to rule out persistent Müllerian duct syndrome (PMDS) due to an AMH mutation. Testosterone and gonadotropin assays are also helpful in this regard.

Isolated AMH Insufficiency: The Persistent Müllerian Duct Syndrome

Most types of DSD are characterized by testosterone dysfunction, isolated or not. The persistent Müllerian duct syndrome (PMDS) is the only example of DSD due to an isolated defect of AMH synthesis or action (**Figure 4G**). PMDS is a rare autosomal recessive disorder characterized by the persistence of Müllerian derivatives, uterus, and Fallopian tubes, in otherwise completely virilized 46,XY males. Bilateral cryptorchidism is observed most frequently: the uterus remains anchored to the pelvis and mechanically prevents testicular descent because it is tethered

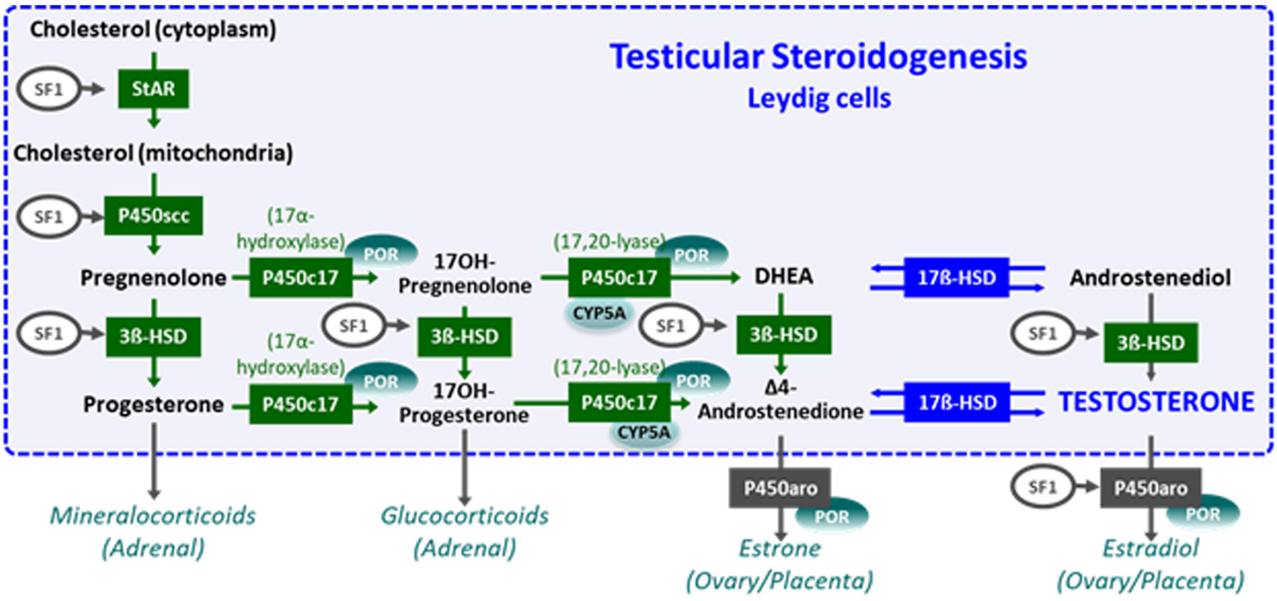

FIGURE 5 | Steroidogenesis steps in testicular Leydig cells (within the dotted blue box) and in the adrenals, ovaries, and placenta (outside the box). Reproduced with permission from (49). Copyright© 2020 Grinspon, Bergadá and Rey.

to the testes by the male excretory ducts. Alternatively, one or both testes may make it into the inguinal canal or the scrotum, dragging the uterus along. This may result either in unilateral cryptorchidism with a hernia containing the uterus on the opposite side, a condition known as "*hernia uteri inguinalis*." The testis on the opposite side can be drawn into the same hemiscrotum by gentle traction or may already be present there; this condition typical of PMDS is named "transverse testicular ectopia." The anatomical picture may vary within the same family and is not correlated with the genotype (64).

The condition is due to mutations of either the AMH or AMHR2 gene (65). Molecular diagnosis has now been achieved in nearly 200 patients worldwide. Serum AMH is usually very low or undetectable in mutations of the AMH gene, even those affecting the inactive N-terminal proregion. In contrast, the AMH serum level does not significantly differ from control values in mutations of AMHR2 or in idiopathic cases, in which no genetic abnormality of either AMH or AMHR2 has been detected, but this is becoming rarer as new-generation massive parallel sequencing is gaining ground.

In practice, diagnosis of PMDS poses few problems in familial cases or in patients with *hernia uteri inguinalis* or transverse testicular ectopia, both very evocative. In patients with impalpable testes, other disorders must be ruled out such as simple bilateral cryptorchidism, anorchia, or even Prader V congenital adrenal hyperplasia, assuming karyotype analysis has not been performed. Paradoxically, in this regard, AMH assay is of little value because according to the gene involved, serum AMH can be either undetectable as in anorchia and CAH or normal or in the lower range of normal, as in cryptorchidism. Ultrasound pelvic examination to display Müllerian organs is the diagnostic method of choice. AMH assay is useful only to orient molecular investigation.

THE 46,XX CHILD WITH DSD

Complete or partial virilization of an XX individual is due to the production of androgen, either from testicular tissue or from other sources: the fetal adrenal, the placenta, or very rarely an ovarian tumor in the mother. Adrenals, placenta, and ovaries do not synthesize AMH, at least not in significant amounts. Thus, AMH assay can easily detect the presence of testicular tissue in such patients.

46,XX DSD With Testicular Tissue

Testes are not expected to differentiate in the absence of a Y chromosome, with possible exceptions. SRY may translocate from the Y to X chromosome during paternal meiosis (66, 67), an XY cell line may lurk in gonadal tissue, or a mutation or copy number variations in critical genes may upset the fragile equilibrium between protestis and proovary genetic pathways [reviewed in (68)]. Serum AMH above the low levels expected for a female sound the alert if the phenotype is ambiguous (**Figure 4E**).

XX males are endowed with bilateral testes and are usually fully virilized (**Figure 4B**); in most, but not all cases, Y material can be detected in their DNA, usually located on an X chromosome, but a minority of XX males are SRY negative and often sexually ambiguous. XX males resemble Klinefelter boys insofar as childhood is usually uneventful, XX germ cell degeneration sets in at puberty, and infertility is inevitable. AMH

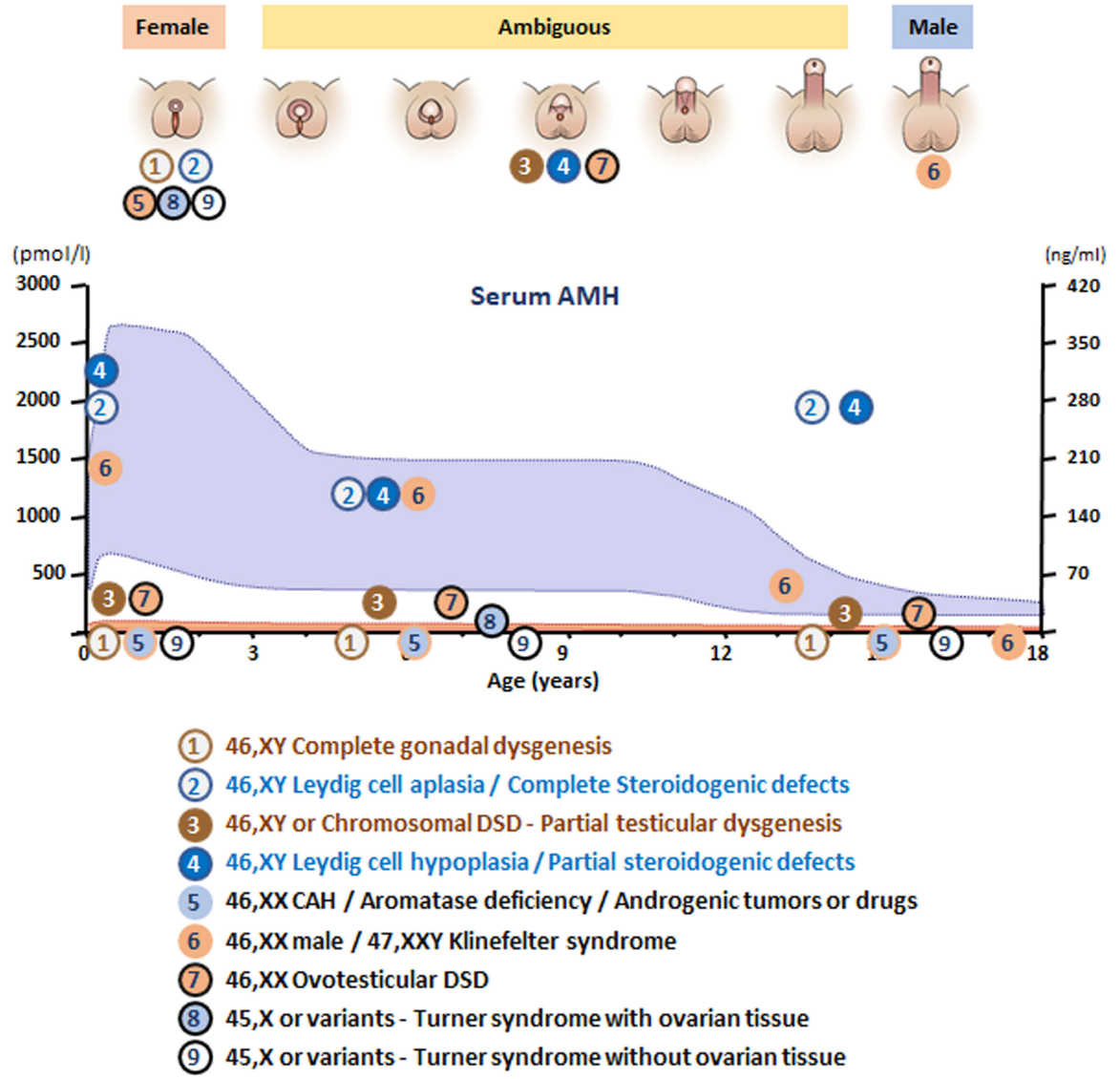

FIGURE 6 | Schematic of AMH levels in various types of disorders of sex development (DSD) in relationship to the aspect of the external genitalia and age. The shaded area represents reference levels for AMH, as obtained from ref. (22).

is in the normal range during childhood and falls subsequently (6 in **Figure 6**).

Ovotesticular DSD is a multifaceted condition defined by the coexistence in the same individual of testicular and ovarian tissue, either separate or associated within an ovotestis. External genitalia can be more or less virilized, but internally, a uterus is present in 70% of cases (69) and ovarian tissue is functional whereas testicular tissue is usually dysgenetic. An AMH serum level above normal female values in a sexually ambiguous XX baby is a strong argument for the diagnosis (7 in **Figure 6**). Values approaching male standards are found only during the first 2 weeks after birth in patients with large amounts of testicular tissue (35); later, AMH concentration decreases in keeping with the progressive degeneration of testicular tissue (69). Most karyotypes are 46,XX or 46,XX/46,XY chimerisms (**Figure 4E**). A 45,X/46,XY mosaic should awaken suspicion of mixed or asymmetrical gonadal dysgenesis, in case an undifferentiated streak with "ovarian stroma" has been mistaken for genuine ovarian tissue (70).

46,XX DSD Without Testicular Tissue

In this context, the androgens responsible for virilization of the external genitalia come from an extra-testicular, source but in the absence of Sertoli cells, AMH is not produced. **Congenital adrenal hyperplasia** (CAH) due to 21-hydroxylase deficiency accounts for more than half the cases of DSD and is the first diagnosis evoked by a pediatrician confronted with a sexually ambiguous or apparently male baby with impalpable testes.

Aromatase deficiency, a rare condition due to a mutation in placental cP450arom (71), should only be considered in an XX sexually ambiguous child once CAH has been ruled out, and AMH is in the female range, thus ruling out ovotesticular DSD. Maternal ovarian tumors, for instance hCG-dependent **luteomas**, are another rare cause of XX DSD (72). Exogenous androgen or progestin administration during pregnancy, as a cause of fetal virilization, is extremely rare nowadays (73). In all these conditions, AMH serum values are in the female range (5 in **Figure 6**), uterus, Fallopian tubes, and ovaries are normal.

SEX CHROMOSOMAL DSD

Sex chromosomal DSD designate conditions where sex chromosomes are neither uniformly XX nor XY. Mosaicisms or chimeras with at least one Y-chromosome lineage are usually associated with DSD due to mixed or asymmetrical or ovotesticular dysgenesis. The AMH level in serum is grossly correlated with the amount of functioning testicular tissue present (35).

Turner Syndrome

Women with Turner syndrome have different karyotypes, all of which lack X chromosomal material; a 45,X karyotype is present in 40–50% of cases, 45,X/46,XX in 15–25% (74). Patients experience accelerated loss of ovarian follicles starting in fetal life and inexorably leading to ovarian insufficiency and infertility at various ages (75, 76). Preservation of fertility may be feasible by cryopreservation of ovarian tissue before follicles have totally vanished. AMH assay, often considered a marker of ovarian reserve, is detectable in a fifth of Turner girls (8 in **Figure 6**), essentially those with a mosaic 46,XX cell line, indicating that follicles are still present and cryopreservation is still a viable option.

Klinefelter Syndrome

Klinefelter syndrome is the most common form of hypogonadism in males. It is characterized by a supernumerary X chromosome; the classical 47,XXY karyotype is present in 90% of cases. Cryptorchidism and mild developmental disorders may alert to the diagnosis in childhood, but usually patients are referred in adulthood for infertility (77). During childhood, AMH serum level is in the normal range during childhood, decreasing to lower levels after puberty (78, 79), indicating that Sertoli cell function is not impaired before mid to late puberty (6 in **Figure 6**).

CONCLUDING REMARKS

AMH is a reliable biomarker of testicular and ovarian function and is extremely useful for the differential diagnosis of DSD (**Figure 6**), especially when confronted with serum testosterone concentration. Sertoli cells of the testes produce high amounts of AMH from early fetal life until the onset of puberty, while granulosa cells of the primary and small antral ovarian follicles produce small amounts of AMH from late fetal life until menopause. In patients with DSD, serum AMH is useful for the following purposes: (a) detect the existence of testicular tissue; (b) determine the amount of functional Sertoli cells; (c) distinguish between congenital disorders affecting whole testicular differentiation (gonadal dysgenesis) and those affecting exclusively Leydig cells or Sertoli cells; (d) direct the diagnosis of PMDS to the study of the *AMH* or the *AMHR2* gene; (e) detect the existence of ovarian follicles and the possibility or preserving fertility in girls with Turner syndrome; and (f) assess Sertoli cell function in boys with Klinefelter syndrome.

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REVIEW
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Use of AMH in the Differential Diagnosis of Anovulatory Disorders Including PCOS

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Since the historical use of gonadotrophin and estradiol levels to define the different anovulatory disorders has shown some limitations, the use of other markers such as anti-müllerian hormone (AMH) has been proposed. This review addresses the role of AMH in the differential diagnosis of anovulatory disorders, especially focusing on its value in the prognostic characterization of their severity. Current limitations and future clinical applications are discussed.

Keywords: anovulatory disorders, polycystic ovary syndrome, amenorrhea, anti-Müllerian hormone, premature ovarian insufficiency

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INTRODUCTION

Anovulatory disorders in women can be various. The main classification in use is adapted from the one proposed by the World Health Organization (WHO) and by the 1995 ESHRE Capri workshop group (1). The possible causes of anovulation are here categorized into three groups on the basis of serum gonadotrophin and estradiol levels. Besides having diagnostic purposes, such classification also aims at guiding the therapeutic approach, since each anovulation subtends different long term health consequences and ovulation restoring strategies. WHO 1 anovulatory dysfunction, which accounts for 5–10% of all anovulatory disorders, is characterized by low gonadotrophin and low estradiol serum levels (2). The underlining cause of this dysfunction is usually a hypothalamic suppression, which occurs in association with weight loss and negative energy balance, such as in patients suffering from anorexia nervosa and endurance athletes. WHO 2 anovulatory dysfunction, which accounts for the 80% of all the ovulatory disorders, presents normal gonadotrophin and estradiol levels. Polycystic ovary syndrome (PCOS), which is diagnosed on the basis of the Rotterdam consensus criteria (at least two between oligo-anovulation, clinical or biochemical hyperandrogenism and polycystic ovarian morphology, PCOM), represents its most frequent example (3). At last, WHO 3 anovulatory dysfunction is characterized by an ovarian reserve depletion with high gonadotrophin and low estradiol levels. If the patient is younger than 40 years old, this may indicate premature ovarian insufficiency (POI) (4).

Gonadotrophin and estradiol levels, however, are often overlapping in the various forms of anovulation, with no clear discriminatory thresholds established. Apart from their use, which can be limited by the above mentioned vagueness, several other markers have been proposed for the differential diagnosis of anovulatory dysfunctions. In particular, serum anti-Müllerian hormone (AMH) levels have been indicated as a potential tool in the discrimination of the various

anovulatory disorders. In this article we aimed at reviewing the literature on AMH as a differential diagnostic marker in anovulatory diseases.

AMH FUNCTION IN THE OVARY AND AT THE CENTRAL LEVEL

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein belonging to the transforming growth factor-beta (TGF-β) superfamily (5). In men, AMH is secreted by the Sertoli cells of the testes, inducing the regression of Mullerian ducts (6, 7). In adult life, AMH is exclusively produced in the ovary by the granulosa cells surrounding the growing follicles, from early antral to small antral follicles phase (8–10). It is therefore thought that its serum levels are a reflection of a cohort of small growing follicles (11, 12), which reflects the number of residual primordial follicles, or the ovarian reserve (13).

AMH is a key-regulator of ovarian function. It is considered a local growth factor and acts in the cellular differentiation, since it has been demonstrated to have a paracrine inhibitory effect on the activation of folliculogenesis (5). *In vitro* and *in vivo* studies on mice were the first to show that in AMH knockout animals the transition from primordial into growing follicles with subsequent early depletion of the primordial follicle pool was enhanced (13). In humans, AMH causes a decrease in the follicle-stimulating hormone (FSH)-stimulated aromatase expression (14), and also reduces FSH receptor messenger RNA (mRNA) expression (15), with a consequent modulation in the ovarian follicular responses to gonadotrophins. Moreover, *in vitro* studies proved a modulation of the response to luteinizing hormone (LH) induced by AMH (5). A central action of AMH on GnRH neurons has also been hypothesized in mice observing the increased LH pulsatility in many cases of PCOS, in which circulating AMH levels are also often elevated (16).

AMH IN WHO 1 OVULATORY DYSFUNCTION

In WHO 1 patients, either low, normal or slightly elevated AMH levels have been described.

Normal serum AMH levels have been reported in most of the studies on women with central secondary amenorrhea (17–20). La Marca et al. showed that in women with functional hypothalamic amenorrhea, AMH serum levels were similar to those found in normal controls. Moreover, there were no significant differences between the two groups in the number of 2–6 mm follicles, suggesting that initial follicle recruitment is not abolished in hypogonadotropic hypogonadism, with a stagnation of small antral follicles (17). No statistically significant difference of AMH serum levels between women with hypothalamic amenorrhea and anorexia nervosa and control group was observed in another case-control study conducted by Luisi et al. (18). Levels of AMH were within the normal range for age in a further study of van Elburg et al. on patients suffering from anorexia nervosa, confirming unaffected gonadotropin-independent growth of small preantral and early antral follicles in these patients. Under the same conditions of initial body weight, premorbid weight, duration of amenorrhea, duration of study participation and amount of prescribed medications, in patients suffering from anorexia nervosa the higher the AMH levels, the higher the probability of ovarian function recovery, indicating a possible prognostic role for AMH (21). The same evidence was observed in a group of seven patients with hypopituitarism during the years of adolescence, whose AMH serum concentrations were in the age-specific reference range, while in three out of four patients diagnosed in the infancy AMH serum concentrations were on or below the age-specific 25th percentile, with worse prognostic implications (22). On the contrary, in patients with long term profound gonadotrophin deficiency such as isolated hypogonadotropic hypogonadism and Kallmann syndrome, AMH levels were significantly lower when comparing affected patients to healthy controls. The subgroup of patients with the lowest FSH levels showed also the lowest AMH levels, showing the correlation between AMH levels and the severity of gonadotropin deficiency (23).

In a recent study by Alemyar et al., median AMH values were significatively higher in a population of patients with hypothalamic hypogonadism compared to healthy controls, but lower than AMH levels in the PCOS population. We hypothesize that the increase in AMH levels in the group of patients with hypothalamic hypogonadism might be due to the presence of a relatively large pool of antral follicles smaller than 2 mm in diameter, which are not counted during transvaginal ultrasonography although they secrete AMH (24).

AMH IN WHO 2 OVULATORY DYSFUNCTION

Polycystic ovary syndrome (PCOS) represents the most frequent clinical manifestation of WHO 2 anovulatory dysfunction. Serum AMH is consistently higher in PCOS women (17, 25–30). For such reason, since serum AMH levels reflect the excess of small follicles which ultrasonography cannot detect, AMH has been proposed as a better marker in the diagnosis of PCOS than antral follicle count (AFC) (31–34). In a meta-analysis conducted by Iliodromiti et al., the specificity and sensitivity in diagnosing PCOS in the symptomatic women were of 79.4 and 82.8%, respectively, for a cutoff value of AMH of 4.7 ng/mL (31). Dewailly et al. separated asymptomatic women with PCOM to those with normal ovarian morphology in order to better calibrate the cutoff for the AMH value to distinguish patients with PCOS from normal women. A higher specificity (97 vs. 92%) and a better sensitivity (92 vs 81%) were demonstrated for a cutoff value of AMH of 4.9 ng/mL compared to AFC (32). Nevertheless, a universal diagnostic threshold for serum AMH in the diagnosis of PCOS has not yet been reached, and its use as an alternative for detecting PCOM in the diagnosis of PCOS has

not been recommended by the new European Society of Human Reproduction and Embryology 2018 guidelines (35).

The reasons behind the elevated AMH levels of these categories of patients are different. In PCOS women there is a stagnation of AMH-producing follicles, with a stockpiling of transitional and classic primary follicles whose differentiation in the subsequent development phases is disrupted (36). Besides the elevated number of AMH-producing follicles, in these patients an increased production of AMH per single follicle has also been observed, with the mean level of AMH four times higher in granulosa cells from ovulatory PCOS and 75 times higher in granulosa cells from anovulatory PCOS patients in a study by Pellatt et al., demonstrating a correlation between AMH values and the severity of the syndrome with the possibility of ovulation restoration (37). Besides being a marker for the diagnosis of PCOS, AMH has also been thought to have a role in the pathogenesis of the disease. Both Visser and Homburg stated that the high AMH concentrations present in women with PCOS could play an integral role in causing anovulation due to AMH's inhibitory influence on the actions of FSH that normally promotes follicular development from the small antral stage to ovulation (38, 39). According to Cimino et al., AMH increases GnRH-dependent LH pulsatility and secretion, with the consequent dysregulation of follicle growth (16). Moreover, AMH seems to correlate with the severity of the syndrome. Higher levels of AMH have been shown in amenorrhoeic than in oligomenorrhoeic women with PCOS, reflecting a more evident impairment in follicular development and granulosa cell function in the ovaries of amenorrhoeic than in those of oligomenorrhoeic PCOS women (17). According to Tal et al., increased AMH levels correlate more with PCOS severity in women with ultrahigh AMH (> 10 ng/mL) having greater prevalence of polycystic ovarian morphology, oligomenorrhea and amenorrhea than in women with AMH 5–10 ng/mL (40). Nevertheless, AMH has also been proposed as a marker for treatment monitoring in PCOS women. PCOS obese and overweight patients who showed improvements in reproductive function after weight loss had lower baseline AMH levels compared with those who did not respond (41). In a study evaluating response to treatment, the group who responded less well to induction of ovulation was the one with higher AMH levels (42).

AMH IN WHO 3 OVULATORY DYSFUNCTION

WHO 3 anovulatory dysfunction is characterized by low or undetectable AMH levels (17, 43, 44). Being connected to the loss of ovarian function, this condition is also known as primary ovarian insufficiency (POI), premature ovarian failure (POF) or premature menopause (44) when this condition occurs before age 40. In such dysfunction, the follicle pool is depleted, with a consequent ovarian insufficiency whose cause can be various: genetic, autoimmune, and iatrogenic (2). With respect to other markers such as AFC, FSH, inhibin B and estradiol, AMH seems to better reflect the continuous decline of the oocyte/follicle pool with age (15). AMH levels showed to be significantly different between incipient ovarian failure (IOF), with regular menstrual cycles and elevated FSH, and transitional ovarian failure patients (TOF), with oligomenorrhea and elevated FSH, permitting the identification of the clinical degree of follicle pool depletion (45). AMH values are also reported to perform as a predictor of follicle presence in ovarian biopsies performed on patients with a premature ovarian failure (46). Moreover, in patients diagnosed with steroidogenic cell autoimmunity (SCA-POI), since the depletion of follicles begins from the antral follicle stage, a preserved ovarian follicle pool producing AMH can be found for several years after an ovarian insufficiency diagnosis (47). The maintenance of AMH levels in women with autoimmune POI is important since it has been observed that in the initial stages of the disease, characterized by the persistence of antral follicles despite amenorrhea and high serum gonadotropin levels, strategies of fertility preservation such as *in vitro* maturation (IVM) can be performed before the inevitable follicular depletion (48).

DISCUSSION

The Anti-Müllerian hormone (AMH), given its relationship with the follicular ovarian pool, is a reliable marker of ovarian reserve and its clinical use has recently been extended and emphasized. In particular, it has been proposed by different authors as a potential marker in the differential diagnosis of the various forms of anovulatory dysfunctions: it is usually normal in patients with hypogonadotropic anovulation (even if it can be also low or slightly elevated), high in normogonadotropic anovulations and low in hypergonadotropic anovulations (2, 7, 15). Due to its significantly elevated values in PCOS it has also been proposed as a marker for the diagnosis (31–34), even if a universal diagnostic threshold for serum AMH in the diagnosis of PCOS has not yet been reached (35).

Its use as a single marker in differentiating the different anovulatory forms, however, has been discussed. Since its levels can be increased in patients with hypothalamic hypogonadism whilst there is no increase in AFC, Alemyar et al. warned to avoid its use as a single biomarker for the characterization of an anovulatory disorder, highlighting the importance of measuring gonadotrophins and estradiol in order to avoid the misdiagnosis with WHO 2 anovulations (24). Nevertheless, AMH has proven to be insufficient in characterizing alone some anovulations such as WHO 1 anovulation disorder, since in such anovulation its values can vary widely. However, the underlying causes (congenital, functional or iatrogenic) of WHO 1 anovulation are not always specified in the literature, which can represent an important limitation. The evaluation of the differences in serum AMH between long-term vs. short-term etiologies could in the future be helpful in guiding the evaluation of AMH in WHO 1 anovulations.

Several evidences from the literature show that, rather than a diagnostic biomarker, AMH could have an important role as an index of severity and a guide for treatment of the various

anovulatory disorders. A correlation between AMH values and the probability of ovulation restoration has been described, even if, to date, no predictive values in terms of ovulation and pregnancy for any ovulation-inducing treatment have been demonstrated for this assay.

An important limitation in the use of AMH as a single diagnostic marker in the differential diagnosis of anovulations is represented by different numerical calibration of the existing AMH assays. An international standard is needed to standardize the existing assays before diagnostic cutoffs are meaningful. The optimal performance and stability of the automated AMH assays now in use compared to previous manual assays is well recognized (49, 50), with many clinicians assuming that the values derived from the two most common automated assays, the Elecsys AMH assay and the Access AMH assay, are interchangeable (51). Nevertheless, a certain debate is still present in literature (51, 52). Another issue is the change of serum AMH level with age in the normal population, and hence an age-standardized value may be more appropriate in serving the diagnostic role. Defined study and control populations, biologically relevant cutoff values that reflect clustering of clinical features and are relevant to health outcomes and age-specific and improved accuracy and standardization of AMH assays are necessary before introducing AMH values as a diagnostic marker for PCOS (53).

In conclusion, AMH has been reported as a relevant diagnostic marker for anovulatory disorders. A debate in literature has been conducted over its candidability as a diagnostic marker in WHO 2 anovulations. However, at the moment its use for such purpose has not been recommended, while its employment in the characterization of the prognosis of the anovulations could be an important field of research and clinical application. Improvements in standardization of AMH assays and establishment of cutoff values based on large-scale validation in populations of different ethnicities and ages are needed.

AUTHOR CONTRIBUTIONS

MC did the bibliographic research and wrote the article. ALM revised the manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Anti-Müllerian Hormone in the Pathogenesis of Polycystic Ovary Syndrome

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Besides its interest for diagnosis, the finding of an elevated serum AMH level in PCOS has open major pathophysiological issues. This review addresses the three most important issues: 1- the role of AMH in the disturbed folliculogenesis of PCOS; 2- the role of AMH in the gonadotropin dysregulation of PCOS and 3- the role of AMH in the trans-generational transmission of PCOS. For each of those issues, the clinical and experimental evidences currently available are discussed and pathophysiological hypothesis are proposed.

Keywords: anti-müllerian hormone, polycystic ovary syndrome, androgens, follicle, FSH, anovulation, GnRH, aromatase

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INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of childbearing age and the leading cause of hyperandrogenism (HA) and oligo-anovulation (OA) causing infertility (1). PCOS is characterized by an increased number of ovarian follicles at all growing stages (2–4). This increase is particularly seen in the pre-antral and small antral follicles. Interestingly, it is precisely those follicles that primarily produce AMH (5, 6). Release of AMH from the granulosa cells (GCs) of antral follicles leads to measurable serum levels, and these concentrations have shown to be proportional to the number of developing follicles in the ovaries. The development of sensitive assays has enabled measuring AMH in serum and its level was found 2–4 fold higher in women with PCOS than in healthy women, as detailed in other articles of this series. This elevated serum AMH level was initially considered a reflection of the increased stock of pre-antral and small antral follicles within polycystic ovaries (PCO) (7, 8). In addition, it could also result from an increased production of AMH per follicle (9), due to an intrinsic property of GCs in PCO that will be discussed below.

The elevated serum AMH level in PCOS quickly interested PCOS specialists who saw it as a way of circumventing the heterogeneity of the ultrasound description of polycystic ovarian morphology (PCOM) that is used in the definition of PCOS. Indeed, the antral follicular count (AFC) being very dependent on the material used, some authors investigated the diagnostic value of the serum AMH assay as a surrogate for follicle number per ovary (FNPO) [reviewed in (10)]. Finally, marking the excess of antral follicles in women with polycystic ovary syndrome (PCOS), AMH assay may soon replace and/or complete the ultrasound ovarian morphology criterion in the diagnosis of this syndrome (11).

Besides its interest for diagnosis, the finding of an elevated serum AMH level in PCOS has open major pathophysiological issues. First, attention has been drawn to its positive association with hyperandrogenism (HA) (7, 8), whom mechanisms are discussed in this review. Then, studies have shown correlation with the PCOS phenotypes, as defined by the Rotterdam criteria (phenotype A: amenorrhea or oligomenorrhea + HA + PCOM; phenotype B: amenorrhea or oligomenorrhea + HA; phenotype C: HA + PCOM; and phenotype D: amenorrhea or oligomenorrhea + PCOM). The highest serum AMH levels are found in phenotype A (12). Conversely, mean AMH serum levels were found to be lower in hyperandrogenic eumenorrheic patients (phenotype C) compared to those with amenorrhea or oligomenorrhea (13), even if they were not hyperandrogenic (phenotype D) (14). This could mean that the AMH excess is the hallmark of a GCs deregulation that plays a major role in the anovulation of PCOS, besides other contributors such as hyperandrogenism and/or excessive LH secretion and/or hyperinsulinism (15). We will discuss this important issue.

Besides the primary autocrine role of AMH in the deregulation of GCs of PCO, the recent discovery of the AMH receptor in a significant subset of GnRH neurons suggests possible extragonadal effects of AMH on the hypothalamic-pituitary-gonadal axis (16) that might be exacerbated in PCOS. Finally, recent data suggest that AMH could be involved in the epigenetic re-programming that is now believed to be the main mechanism leading to PCOS at puberty and adulthood (17). The goal of this review is also to discuss this exciting issue.

ROLE OF AMH IN THE DISTURBED FOLLICULOGENESIS OF PCOS

Is AMH Overexpressed at the Follicle Level?

The hypothesis of a role for AMH in follicular deregulation of PCOS assumes that the expression of this hormone is exaggerated within each follicle and/or that its signaling pathways are amplified. This is difficult to demonstrate *in vivo* because the excess of growing ovarian follicles (up to the stage of small antral follicles) in women with PCOS (2) is a confounding factor. Indeed, this alone could explain the rise in AMH levels because it is those follicles that physiologically secrete AMH (18). In addition, a close correlation has also been shown between plasma AMH levels and the excess of 2–5 mm of antral follicles on ultrasound (8). Thus, it is accepted that the increase in granulosa "mass" secondary to the excess of growing follicles explains at least in part the excess plasma AMH level in women with PCOS (10, 19, 20).

Another explanation, not excluding the first, could be an excess secretion of AMH intrinsic to the growing follicles of women with PCOS (9, 20, 21). Some authors have reported a significant increase in the AMH/AFC ratio in women with PCOS compared with women with asymptomatic ultrasound PCO and non-PCOS controls (6, 22). This suggests a probable over-expression of AMH by the GCs from antral follicles in PCOS women.

In agreement, Pellatt et al. (23) demonstrated *in vitro* in GCs cultures from oophorectomy specimens that the AMH concentration in the culture media was 4 times higher in normo-ovulatory PCOS women and 75 times higher in anovulatory PCOS women compared with GCs from control women. *In vivo*, Das et al. (24) highlighted that the AMH concentration in follicular fluid of 4 to 8 mm antral follicles, was 5 times higher, outside of any ovarian stimulation setting. Catteau-Jonard et al. (25) demonstrated increased transcription of the AMH gene and its receptor by quantitative RT-PCR on partially luteinized GCs collected during oocyte puncture for *in vitro* fertilization in women with PCOS, compared with control women. This increased transcriptional activity was seen in both selected intermediate-sized (8–13 mm mean diameter) and larger dominant follicles (17–22 mm mean diameter). All these data suggest increased expression of AMH by the GCs of women with PCOS, probably secondary to intrinsic dysfunction of these cells.

Nevertheless, all teams do not validate this hypothesis. Owens et al. (26) found no difference in the transcription of the AMH gene or its receptor in their study which compared the expression of 13 genes by quantitative RT-PCR by GCs from small, unstimulated antral follicles (on ovarian cortex sampled for fertility preservation) and on partially luteinized GCs (in patients benefiting from *in vitro* fertilization) in women with PCOS vs. control women. On the other hand, Dilaver et al. (27) found no basal increase in the expression of AMH transcripts in cultured GCs from PCO compared with normal ovaries. These results should nevertheless be put into perspective, given the low level of AMH expression in cultured human GCs and the small number of cases studied.

Another explanation at the molecular level could be an increased stability of the messenger RNAs resulting from the transcription of the AMH gene in the GCs of PCOS women. Thus, even if the transcription of the AMH gene is not increased, an exaggerated stability of the messenger RNAs could lead to a more marked translational activity and thus to an increase in AMH secretion. The degree of polyadenylation of the 3′-untranslated regions (3′-UTR) of mRNAs coding for AMH could be one of the explanations. However, this hypothesis has not been the subject of any specific study to date.

Finally, the role of certain microRNA which are known to be inhibitors of the translation of messenger RNAs, could also be mentioned. Nevertheless, the only study available to date has not been able to confirm this hypothesis.

If It Is Real, What Is the Explanation for the Excess Production of AMH by GCs From PCO?

At the molecular level, no abnormalities in the AMH gene that could lead to excess transcription have been reported in PCOS women (28). A whole series of studies point to the responsibility of hyperandrogenism, but controversy persists as to the reality of this effect and its mechanisms, which may be direct or indirect.

In vivo data in PCOS patients are contradictory. A possible direct stimulatory effect of androgens on the expression of AMH by GCs was first raised when a positive correlation between

serum AMH and androgen concentrations was reported in several series of PCOS women (7, 8, 29–31). However, many confounding factors may play a role, in particular the positive effect of androgens on the number of growing follicles (32) and thus on the "granulosa mass." Caanen et al. (33) observed that administration of androgens as part of female-to-male transitions induced a significant decrease in AMH levels, but the protocol included the use of a GnRH agonist, which might have confused the results by lowering serum FSH level (see below). Finally, the decrease in serum AMH levels in PCOS patients receiving high-dose cyproterone acetate, a progestin with a potent anti-gonadotropic and peripheral anti-androgenic action, was no greater than under other anti-gonadotropic drugs, such as estrogen-progestin contraceptives (34, 35). But here again, serum FSH level is low in these situations.

Similarly, *in vitro* experimental data are contradictory. An androgen-inhibitory effect of androgens on the secretion of AMH by Sertoli cells in men has been clearly demonstrated for many years (36). Crisosto et al. (37) demonstrated that high-dose testosterone was responsible for decreased levels of AMH expression in GCs from small bovine follicles. On the contrary, Zhang et al. (38) reported that testosterone caused an increase in AMH mRNA levels in GCs from mouse antral follicles. In women, some authors have not demonstrated any effect of 5α Dyhydrotestosterone (DHT) on the expression of AMH in GCs from control patients, whereas an increase was observed only in GCs from PCOS patients (39). Dilaver et al. (27) also observed this dose-dependent effect of DHT, while that of testosterone was either positive or null according to its concentration in the GCs culture medium. It should be noted that the contradictory results between these different studies on the effects of androgens on the expression of the AMH gene could be explained by the great variability of the models used (different animal species, cell type, analysis method). Moreover, the effect of androgens is to be seen in the complex interactions they have with other important actors at the GCs level, such as FSH and Estradiol (E2), which vary according to the follicular stage and which are not always taken into account in experimental studies (**Figure 1**) (19).

Several studies suggest an indirect effect of androgens, via an increase in the number of FSH receptors (FSHR) and/or estradiol receptors alpha (ERα). Many studies converge toward the promoting action of androgens on the transcription and translation of FSHR through genomic and non-genomic effects and this effect is likely enhanced in PCO [reviewed in (19)]. Consequently, the stimulating effect of FSH on AMH expression that occurs in small growing follicles from normal ovaries would be amplified in PCO (40). This can occur as long as follicles do not express aromatase as E2 inhibits AMH expression through its receptor ERβ (41, 42) (**Figure 1**).

This last phenomenon might be defective in GCs from PCO. Dilaver et al. (27) reported that excess androgens increase the ratio ERα/ERβ, resulting in increased AMH expression. The importance of the relative expression levels of ERα and ERβ has been shown earlier (43). Pierre et al. (39) recently reported a significant positive correlation between the ratio of ERα/ERβ transcripts and the concentration of AMH and an increase in the levels of ERα transcripts in cultured GCs from PCOS women. However, small growing follicles produce very little E2 and this effect of androgens through activation of ERα might not be relevant *in vivo*. Conversely, at the time of large antral follicle selection for dominance and when activation of ERβ is determinant, this effect of androgens maintaining AMH expression might be part of the mechanisms leading to the follicular arrest of PCOS (see below).

Are the AMH Signaling Pathways Normal in the PCO GCs?

In addition to increased expression of AMH, the expression of AMH type 2 receptors (AMHR2) is amplified in PCO GCs (25, 39). Activation of AMHR2 results in a significant increase in phosphorylation of SMAD 1,5,8 in the mouse (44) and of SMAD 5 in luteinized human GCs (45). Intriguingly, Dilaver et al. (27) recently demonstrated in cultured GCs from PCOs a dose-dependent decrease in phosphorylation of SMAD 1,5,8 (P-SMAD 1,5,8) in the presence of AMH, while paradoxically the levels of transcripts of P-SMAD 1,5,8 was increased by about 50% in controls (but without reaching statistical significance). Obviously, if the implication of a deregulation of the AMH signaling pathways in PCOS seems to be an interesting issue, other subsequent studies are needed, especially concerning the involvement of inhibitory SMADs.

What Are the Consequences of the Excess AMH on Ovarian Follicles, According to Their Stages?

Excess AMH Slows Initial Follicular Growth

This hypothesis is based on the seminal experiment of Durlinger et al. (46). The addition of AMH in cell culture media containing follicles from knockout mice for the AMH gene slowed follicular growth, even in the presence of FSH, suggesting an inhibitory effect of AMH on FSH-dependent proliferation of GCs.

In a situation of high AMH such as PCOS, a slowing of the initial "FSH-sensitive" follicular growth could thus occur and contribute to the accumulation of the number of growing follicles within the ovaries in these patients. However, few data specific to the human species have been found to confirm this pathophysiological hypothesis (3).

Excess AMH Decreases Apoptosis of GCs in Small Follicles

Some authors have suggested that AMH has an anti-atretic effect on growing follicles during initial follicular recruitment (27, 47). Some known pro-apoptotic agents, such as vitamin D and leptin, may act by decreasing the expression of AMHR2 and thus the anti-apoptosis effect of AMH on GCs (47).

The data available in the literature for PCOS women are relatively small. Webber et al. (48) demonstrated in cell culture models a lower apoptosis rate of GCs from pre-antral follicles in women with PCOS compared to controls. By immunocytochemistry, GCs from PCO are less and more frequently stained for the markers of apoptosis and anti-apoptosis than in controls, respectively (24, 49). High levels of AMH could be directly involved in this

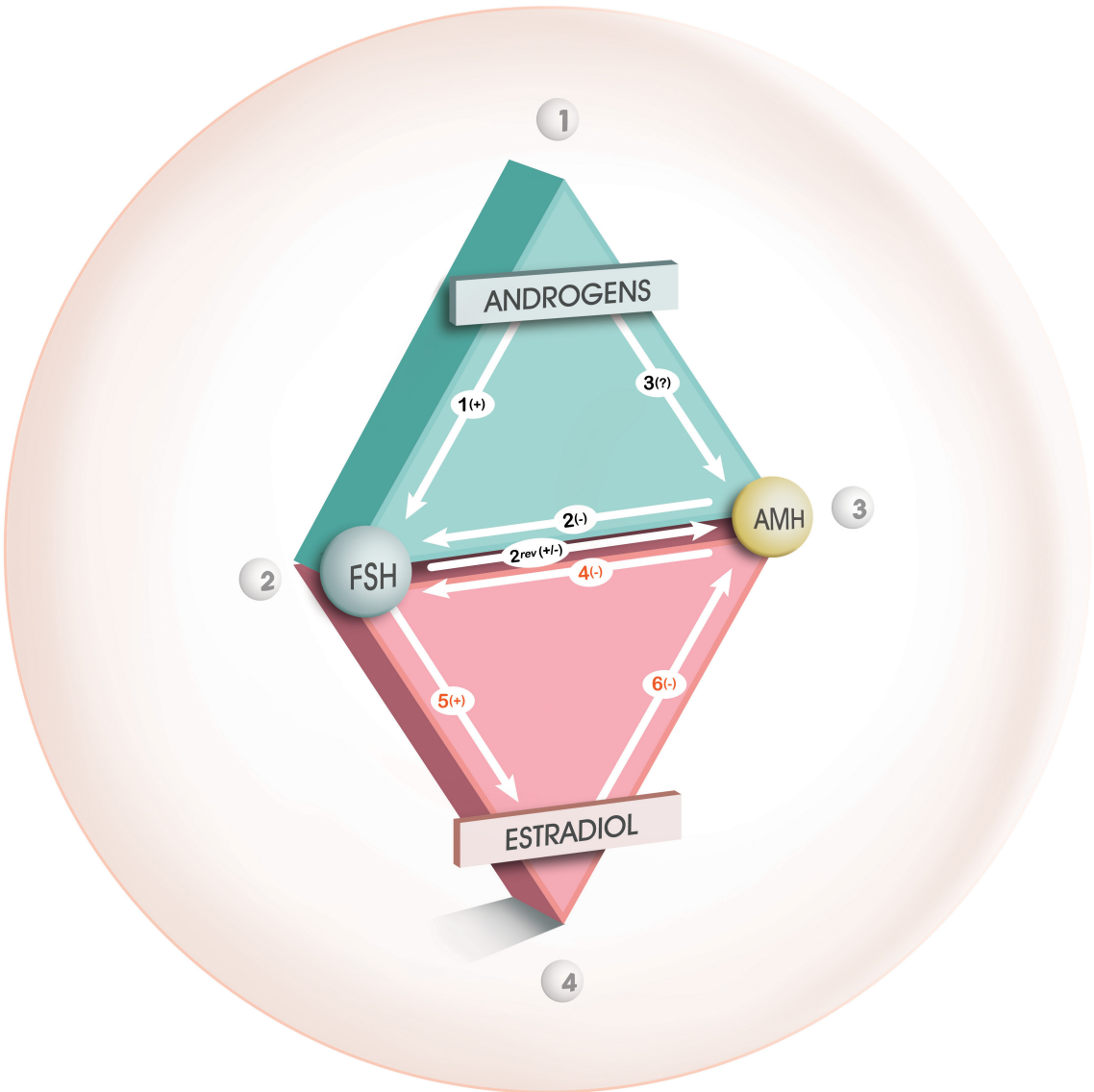

FIGURE 1 | Interaction between androgens, FSH, AMH, and E2 during folliculogenesis. From Dewailly et al. (19), with permission. Relationships between androgens, FSH and AMH during the gonadotropin- independent follicular growth phase (green triangle) and between FSH, AMH and estradiol during the gonadotropin-dependent follicular growth phase (red triangle). "+," "–," or "?" indicate a positive, negative or uncertain effect, respectively, from one of the factors on the other. During the gonadotropin-independent follicular growth phase, the inhibitory effect of AMH mainly influences the promoting effect of FSH on follicular growth (arrow 2). According to our theory, FSH, whose receptors are enhanced by androgens (arrow 1), would stimulate the AMH production during this phase (arrow 2 rev), in the absence of estradiol. A direct effect from androgens on AMH production (arrow 3) is unlikely (see text for details). During the gonadotropin-dependent follicular growth phase, AMH is also involved in a triangular relationship with FSH and estradiol. During this phase, the inhibitory effect of AMH influences mainly the cell differentiation functions induced by FSH (arrow 4), in particular the induction of aromatase (arrow 5). This inhibitory effect will gradually subside, which will allow induction of aromatase by FSH, with consequent synthesis of estradiol which will in turn accelerates the extinction of AMH secretion in large antral follicles (arrow 6).

phenomenon, which would result in a "stock piling" effect (4) contributing to the excess number of growing follicles in the PCOs.

Finally, as menopause approaches, women with PCOS have significantly higher serum AMH levels than non-PCOS women in whom these levels are low or undetectable (50). This may explain why women with PCOS appear to reach menopause at a slightly later age than non-PCOS women (50).

Excess AMH Causes Follicular Arrest in Large Antral Follicles

This phenomenon results from complex interaction between AMH, aromatase, ERs and less likely LH (**Figure 1**).

AMH has been shown to significantly decrease not only FSHR expression but also ovarian aromatase expression [see (19)]. Physiologically, this protects small follicles from premature aromatase expression. When this protective effect of AMH

exceeds its physiological role, because of its excess and/or because it lasts longer than it should, it could lead to a defect in the selection of the dominant follicle, causing what is called "follicular arrest." The fact that AMH inhibits the FSH-dependent factors necessary for follicle dominance adds considerable importance to the elevated serum expression of AMH in PCOS and makes AMH an assumed central player in "follicular arrest." In agreement, it has been shown that the emergence of a dominant follicle in anovulatory women with PCOS on recombinant FSH is preceded by a significant reduction in serum AMH level (51).

In addition, several authors have demonstrated premature expression of the LH receptor (LHR) in GCs of PCOS women. This has been suggested to be the cause of the arrest of follicular growth found in PCOS women with anovulation (52, 53). However, this hypothesis seems unlikely because other authors have more recently demonstrated a negative correlation between the concentration of AMH in the follicular fluid and the expression of the LHR in GCs (54).

The AMH Excess in Follicles Varies According to the PCOS Phenotype

This overexpression of AMH per follicle could vary depending on the PCOS phenotype. Thus, for some authors, in a population of PCOS women, the AMH/AFC ratio was significantly higher in patients with anovulation than in those with an ovulatory phenotype (phenotype C or asymptomatic ultrasound PCO) (22, 55). In contrast, other authors have shown higher AMH levels in hyperandrogenic PCOS women, regardless of ovulatory status (12, 29, 31). The question of variation in AMH expression according to the PCOS phenotype is in fact very complex because principal component analysis has shown that the markers of hyperandrogenism and oligoanovulation are closely related (30). However, when both hyperandrogenism and anovulation are statistically confronted with excess serum AMH, the association is significant with the latter, whereas the former would simply be a confounding factor (23).

To summarize, AMH excess in GCs from PCO would be an indirect consequence of hyperandrogenism and would be involved in the follicle excess of PCO and in the follicular arrest in anovulatory patients.

ROLE OF AMH IN THE GONADOTROPIN DYSREGULATION OF PCOS

A high LH level is found in ∼50% of women with PCOS, with a higher prevalence in women without metabolic impairment (56). It is secondary to the acceleration of the frequency of GnRH secretion which, for some authors, is thought to be the consequence of a negative feedback failure due to prenatal hypothalamic exposure to androgens (57). Conversely, mean FSH levels are lower than controls in many published series, with no precise explanation provided to date. Both phenomena lead to an increase in the LH/FSH ratio, which was used as a diagnostic criterion in the past, but was abandoned because it was too insensitive. AMH could be involved in this disturbance of gonadotropic function.

There Is a Positive Link Between AMH and LH

In women with PCOS, serum levels of AMH and LH are positively correlated (7). This correlation has been shown to be independent of serum androgen and FSH levels (30, 51).

The causal relationship in this relationship has been the subject of debate. For some, the cause would be the high levels of LH that could stimulate AMH secretion and expression as shown by authors *in vitro* from luteinized GCs (23, 58). However, *in vivo*, GCs express LHR late, whereas AMH production begins in primary follicles and peaks before LHR expression (5). Alternatively, recent experimental data suggest that AMH is more likely to have extra-gonadal effects and in particular be capable of increasing the activity of GnRH neurons. The authors have shown that nearly 50% of GnRH neurons (murine and adult human) have specific receptors for AMH type 2 (AMHR2) (16). The combination of several *in vitro* and *in vivo* experiments showed that AMH increased the pulsatile secretion of GnRH-dependent LH through a central action. Indeed, electrophysiological experiments have revealed that exogenous AMH increased the neuronal activity of GnRH neurons; however, this could be an indirect action, as AMHR2 is very widely expressed in the hypothalamic regions, so a synergistic action of other cell types contributing to the increase in GnRH secretion cannot be excluded [for review see (59)]. Similarly, the authors demonstrated that *in vivo* administration of AMH (intracerebroventricularly) was accompanied by a dose-dependent increase in LH secretion and pulsatility. In the end, the increase in AMH concentration would lead to a chain reaction: hypothalamic neurons would start to secrete more GnRH, which would then increase the production and pulsatility of LH by the anterior pituitary gland.

AMH also appears to be able to exert its action at the pituitary level and regulate the activity of gonadotropic cells. It has recently been shown that the expression of the human and mouse AMHR2 gene in gonadotropic cells is regulated by GnRH (60). Indeed, using LβT2 cells, these authors showed that GnRH secreted at a high frequency (1 pulse/30 min) increased AMHR2 expression by gonadotropic cells while a lower frequency (1 pulse/2 h) was without effect. However, the implication of the regulation of pituitary AMHR2 expression as a function of GnRH pulsatility remains to be elucidated in humans and especially in PCOS.

These results raise the hypothesis that the extra-gonadal action of AMH could either be at the origin of, or contribute to, the vicious circle of neuroendocrine and gonadal dysregulation encountered in PCOS.

The Negative Link Between AMH and FSH: A Complex Issue

Low to normal serum FSH levels have long been reported in PCOS (61), even after adjustment for BMI and the number of 2–9 mm follicles (13). Several studies have reported a negative

relationship between serum FSH and AMH levels (8, 51) but no clear explanation has been provided so far. It is unlikely that this reflects a negative effect of FSH on AMH production. In fact, the opposite is suggested by situations of congenital gonadotropic insufficiency where AMH level is decreased and increases under exogenous FSH (62). These contradictory data illustrate the complex relationships between AMH and FSH that may operate at the ovarian and/or pituitary-hypothalamic levels and which vary according to disease state. In the case of PCOS, we hypothesize that by accelerating the pulse frequency of GnRH (see above), an excessive AMH level would increase pituitary secretion of LH to the detriment of FSH (63). It is clear that more attention needs be paid to this issue.

To summarize, new experimental data suggests that AMH is involved in the neuro-endocrine deregulation of PCOS but no human data is available so far to confirm this hypothesis.

IS EXCESS AMH INVOLVED IN THE TRANS-GENERATIONAL TRANSMISSION OF PCOS?

It was in the early 2000s that the hypothesis of prenatal programming of PCOS in relation to gestational hyperandrogenism was first suggested (64). Following this discovery, numerous studies confirmed in various animal models that high testosterone levels during gestation could lead to the appearance of a PCOS phenotype in the offspring (mouse, ewe and non-human primate models) [for review see (57, 65)]. In women with PCOS, the hypothesis of androgen-related prenatal programming is supported by a whole series of studies [see (17) for review], but the origin of this gestational hyperandrogenism remains unknown so far.

Recent studies suggest that AMH may be involved in this phenomenon. Circulating AMH levels are higher in pregnant women with PCOS compared to those with normal fertility (66, 67) and are correlated with androgen levels (67). These results therefore suggest that AMH at relatively high concentration during pregnancy could itself be the cause of prenatal programming of PCOS. This has recently been tested experimentally (66). The authors demonstrated that injection of the bioactive form of AMH (AMHc) into late gestation mice was responsible for the appearance of a hyperandrogenic PCOS phenotype in the offspring in adulthood. In this model, called PAMH, high AMH concentrations during gestation resulted in increased pulsatility of GnRH and LH, which was responsible for gestational hyperandrogenism. Excess maternal LH alone or in combination with AMH would also lead to a decrease in placental aromatase, increasing maternal bioavailable testosterone and causing fetal exposure to androgen excess. This would induce a cascade of events in the offspring leading to an increase in hypothalamic neuronal excitability. In adult offspring, mice show an increase in excitatory afferents responsible for an increase in the excitability of GnRH neurons. The hyperactivity of GnRH neurons then stimulates ovarian steroidogenesis and participates in the vicious circle observed in PCOS by reducing the negative feedback of E2 and progesterone on LH. Prenatal treatment with a GnRH antagonist in PAMH mice prevents the occurrence of the disorders previously observed in the offspring (66). The authors thus demonstrated the predominant role of GnRH, via AMH, in the *in utero* programming phenomenon responsible for the neuroendocrine abnormalities characteristic of PCOS appearing in the offspring.

Finally, it should be noted that this new PAMH mouse model suggests that the maternal hyperandrogenisation observed in PCOS is the result of a central action of AMH on GnRH (and LH) contributing to an increase in ovarian steroidogenesis and an inhibition of placental aromatase expression, leading to an increase in testosterone bioavailability (66). In agreement, continuous administration of a P450 Aromatase Inhibitor induces Polycystic Ovary Syndrome with a metabolic and endocrine phenotype in female rats at adult age (68). In women, inhibition of placental aromatase expression may be the main mechanism in the *in utero* programming of PCOS as serum maternal androgen and LH levels are not as high as in PAMH mice. A decrease in placental aromatase has effectively been observed in women with PCOS who have given birth (69). Studies conducted in mice therefore offer interesting new perspectives that will have to be confirmed in the future by clinical studies in women, since the mouse model is poly-ovulatory and is not perfectly superimposable on the human condition.

To summarize, maternal AMH excess might be one of the causes of *in utero* programming of PCOS, at least in a subset of patients.

CONCLUSION

There is still much knowledge to be acquired to fully understand the pathophysiological role played by the AMH in the PCOS. Clearly, the autocrine action of excess AMH within the GCs is probably the main element of its involvement in the folliculogenesis and anovulation disorder. However, the recent discovery of its endocrine action of retrocontrol on the hypothalamus and the placenta opens up avenues of research likely to lead to new curative or even preventive treatments. For instance, when they are available, antagonists of the AMHR2 might prove to be able to lessen the LH and follicle excess and thus to improve the emergence of a dominant follicle and increase the chances of pregnancy without any ovarian stimulation.

AUTHOR CONTRIBUTIONS

DD contributed to review design, execution, acquisition, analysis and interpretation of data, manuscript drafting, and critical discussion. A-LB, GR, AD, and SC-J contributed to acquisition and interpretation of data, manuscript drafting, and critical discussion. All authors read and approved the final manuscript.

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SYSTEMATIC REVIEW
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The Value of Anti-Müllerian Hormone in the Prediction of Spontaneous Pregnancy: A Systematic Review and Meta-Analysis

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Objective: To determine whether serum anti-Müllerian hormone (AMH) level is a predictor of clinical pregnancy in women trying to achieve a natural conception.

Methods: The PubMed, Embase, and Cochrane Library databases were searched for articles published until August 2020. Studies that met the inclusion and exclusion criteria were included in the meta-analysis; no language limitations were imposed. Quality was appraised using the Quality Assessment of Diagnostic Accuracy Studies-2 checklist. Heterogeneity due to the threshold effect was identified; thus, we plotted a summary receiver operating characteristic curve and calculated its area under the summary receiver operating characteristic curve (AUC) and Cochran's Q index to assess whether AMH level is a predictor of spontaneous pregnancy. Publication bias and sensitivity were also assessed.

Results: Eleven studies (4,388 women) were ultimately included in this meta-analysis. The AUC and Cochran's Q indices were 0.5932 and 0.5702, respectively. For women younger than 35 years, the AUC was 0.6355 and the Q index was 0.6025. For those older than 35 years, the AUC was 0.5536 and the Q index was 0.5403. Subgroup analyses by study type and population characteristics showed results similar to the overall outcome. No publication bias was identified, and the sensitivity analysis confirmed the robustness of the final result.

Conclusions: Serum AMH levels have poor predictive value for natural pregnancy. The predictive value of AMH was poor in the younger and older subgroups. Our findings suggest that low serum AMH levels are not associated with reduced fertility.

Introduction: This study investigated the predictive value of anti-Müllerian hormone (AMH) level for natural pregnancy. Other than age, few factors can predict the chances of natural fertility. AMH is an established biomarker of ovarian reserve that is widely used to predict oocyte yield in cases of *in vitro* fertilization (IVF) and menopause. In clinical practice,

the applications of AMH are increasing. However, its predictive value for natural conception remains controversial. In this study, since AMH is closely related with ovarian reserve, we evaluated whether it has predictive value for natural pregnancy. Our findings will fine-tune the clinical application of AMH in pre-pregnancy counseling. The topic should be of wide interest to investigators in the reproductive endocrinology and gynecology fields.

Systematic Review Registration: PROSPERO 2020 CRD42020216265, Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020216265.

Keywords: AMH, spontaneous pregnancy, meta-analysis, fertility, anti-Müllerian hormone

INTRODUCTION

Female fecundability declines with increasing age due to decreasing oocyte quality and quantity, also known as diminished ovarian reserve (DOR). Age is an independent predictor of ovarian reserve, and females in the late reproductive period usually have a lower chance of spontaneous pregnancy and worse pregnancy outcomes (1). Approximately 10% of women develop a latent ovarian function decline at a younger age, leading to undesirable reproductive outcomes. In such cases, DOR is not clearly identified, and the clinical manifestation shows a regular menstrual cycle but a lower response to ovarian stimulation than that of their peers. Moreover, an ancillary examination shows abnormal ovarian reserve test results. DOR treatment mainly focuses on oocyte quality, oocyte quantity, and fertility (2).

There are several biomarkers of ovarian reserve, such as anti-Müllerian hormone (AMH), follicle-stimulating hormone (FSH), and inhibit-B (3). AMH, a member of the transforming growth factor-β family that is secreted by the granulosa cells of preantral and antral follicles, reflects the follicle reserve and is considered one of the most established biomarkers of ovarian reserve. Compared to other biomarkers, AMH levels are more stable during the menstrual cycle (4). When ovarian reserve starts to decline, serum AMH level changes occur earlier than basal FSH level increases, and menstrual disorders develop. Thus, AMH level is thought to reflect decreased ovarian function early. In clinical practice, AMH level is widely used to predict menopause and reflect ovarian response in cases of assisted reproductive technology (ART) (5–7). However, existing studies have shown inconsistent viewpoints regarding its application in the prediction of natural conception. Several researchers have indicated that clinical doctors should consider AMH levels during fertility counseling, as low AMH levels appear to be a risk factor for a reduced natural pregnancy rate (8–13). However, other studies have reported the opposite result (14–21).

Although numerous case-control and cohort studies have been published to date, high-quality prospective cohort studies stratified by age that fully explore the predictive value of AMH in natural pregnancy are lacking.

Since previous studies presented inconsistent conclusions about AMH for predicting natural clinical pregnancy, this systematic review and meta-analysis aimed to identify whether serum AMH levels can predict natural clinical pregnancy in age-stratified women.

MATERIALS AND METHODS

Search Strategy

This study was performed in accordance with PRISMA guidelines (22). This meta-analysis was registered in PROSPERO (ID: CRD42020216265). The PubMed, Embase, and Cochrane Library databases were searched for articles published until August 2020. The following keywords and subject terms were used: (Pregnancy OR Pregnancies OR Gestation OR Reproductive outcome OR Fertility OR Fecundability OR Conception) AND (AMH OR Anti-Muellerian Hormone OR Mullerian-Inhibiting Hormone OR Mullerian Regression Factor OR Mullerian Inhibiting Hormone OR Mullerian-Inhibitory Substance OR Anti-Mullerian Factor OR Mullerian-Inhibiting Factor OR Anti-Mullerian Hormone). The reference list of each identified primary study was also manually searched to ensure that all eligible studies were included in this meta-analysis. No language-related limitations were imposed.

Eligibility Criteria and Exclusion Criteria

The inclusion criteria were as follows: (i) the study population included women of reproductive age and trying to get pregnant naturally for which the outcomes of clinical pregnancy within a year were recorded; (ii) serum AMH level was measured and study identify a low AMH cutoff value; (iii) women were recruited from the hospital or community; and (iv) sufficient information was available to construct the 2×2 contingency table —the true-positive, true-negative, false-positive, and false-negative test results at certain cutoff values. Considering that infertility is identified as the failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse, studies comparing AMH levels of infertile and fertile women were included as well. If multiple publications reported the same or overlapping data, the most recent study with the largest population was included. If the same population was included in different studies with different selected AMH cutoff values, both studies were included in the meta-analysis. Studies were excluded if populations were restricted to women with diagnosed fallopian tube obstructive infertility, polycystic ovarian syndrome, or autoimmune disease. Reviews, conference abstracts, case reports, and comments were excluded from the study. Studies with insufficient or unavailable data were excluded from the analysis as well.

Study Selection and Data Extraction

Two investigators (CL and MJ) independently screened all titles and abstracts. The full text of the preselected studies was read separately by the same two investigators to identify which met the inclusion criteria. Discrepancies were resolved by discussion with a third investigator (RZ).

The original data were collected separately by the two reviewers to avoid extraction errors. The characteristics of each study were extracted as follows: first author, year of publication, study type, population characteristics, patient ages, suggested AMH threshold (converted to ng/ml using the conversion formula ng/ml = 7.14 pmol/L), AMH assay, and number of true- and false-positive and -negative results. True-positive results were identified as failing to achieve natural pregnancy in a year with a low AMH serum level.

Risk of Bias Assessment

The quality of the selected studies was assessed using RevMan 5.3 according to the QUADAS-2 checklist (23). The risk of bias of each study was divided into low, high, or unclear in terms of patient selection, index test, reference test, and flow and timing.

Data Synthesis

The meta-analysis was performed using Meta-DiSc 1.4 software and STATA 12.0 software. The threshold effect, one of the most important causes of heterogeneity in diagnostic tests, was explored in Meta-DiSc 1.4 (24). The correlation between sensitivity and specificity was calculated to identify the threshold effect (25). A negative correlation (or positive correlation between sensitivities and 1-specificities), which results in a typical pattern of a "shoulder arm" plot in a summary receiver operating curve (SROC) space, suggests that different thresholds or cutoffs used in different studies cause the primary heterogeneity. If the threshold effect was present, the SROC curve was plotted, and its area under the curve (AUC) was calculated with the Cochran's Q index. If no threshold effect is present, diagnostic odds ratio, sensitivity, specificity, and positive and negative likelihood ratios of AMH for predicting pregnancy were also generated. The Chi-square test was further used to explore heterogeneity other than the threshold effect, and the I-squared measure was used to quantify heterogeneity. The test level for the meta-analysis was set at α=0.05. Heterogeneity analyses were performed according to study type and population characteristics (e.g., risk factors for infertility). Deeks' funnel and sensitivity analyses were also performed using STATA 12.0 software to analyze potential publication bias and the robustness of the results.

RESULTS

Study Selection

A flowchart of the study selection process is shown in **Figure 1**. We searched the PubMed, Cochrane Library, and Embase databases and retrieved 4,730 pieces of literature. A total of 3,942 records remained after the removal of duplicates. After careful screening of the titles and abstracts, 110 studies remained and were subjected to the full-text review. In this process, 55 were excluded for the lack of relevance, not meeting the inclusion criteria, or meeting the exclusion criteria; 32 were excluded for article type (25 conference summaries, four reviews, and three clinical study registrations). Of the remaining studies, three did not include sufficient data to make a 2×2 contingency table, seven had unavailable full text, and two included a duplicate population. Finally, 11 studies were included in this meta-analysis (8, 11, 14–16, 26–31).

Study Characteristics

The characteristics, inclusion criteria, and exclusion criteria of the 11 studies are listed in **Tables 1** and **2**. Of them, seven were prospective (8, 11, 14–16, 27, 31), two were case-control studies (15, 30), one was a retrospective cohort study (26), and one was a cross-sectional cohort study (28). Four other studies further included women over the age of 35 years (16, 28, 30, 31), while two focused on only women of younger reproductive age (≤35 years) (11, 14). Among the eligible studies, three included participants with one or more risk factors that may affect natural conception, such as ovarian surgery, endometriosis, or a history of infertility, while five studies did not and three studies compared the serum AMH levels in infertile women or those who achieved a pregnancy after more than 12 months of trying, to those with normal fertility.

Synthesis of Results

Test heterogeneity presented a threshold effect in these 11 studies (n=4,388, Spearman correlation coefficient=0.902, p<0.01). Thus, we plotted an SROC curve and calculated its AUC and Q index, which were 0.5932 and 0.5702, respectively (**Figure 2A**). We further stratified this analysis into age subgroups. Six studies (n=2,908) were included in the young group, with an AUC of 0.6355 and a Q index of 0.6025 (**Figure 2B**). Four studies (n=863) were included in the elderly group, with an AUC of 0.5536 and Q index of 0.5403 (**Figure 2C**). When the studies were categorized by type, seven (n=2,539) were included in the prospective group, while four studies were included in another group (retrospective cohort, case-control, and cross-sectional studies; n=1,849). The AUC was 0.6186 *vs.* 0.5707, while the Q index was 0.5895 *vs.* 0.5531, respectively (**Figures 3A, B**). Among the eligible studies, six included participants with one or more risk factors that may affect natural conception, such as ovarian surgery, endometriosis, and history of infertility (n=1,907), and the AUC was 0.5927 and the Q index was 0.5698 (**Figure 3C**). Five studies included participants without known risk factors that may affect natural conception (n=2,481), with an AUC of 0.6042 and a Q index of 0.5786 (**Figure 3D**).

Risk of Bias of Included Studies

The quality assessment of the included studies is shown in **Figures 4** and **5**. Deeks' funnel plot did not suggest publication bias (p>0.05, **Figure 6**). The sensitivity analysis confirmed the robustness of the calculated results (**Figure 7**). Moreover, a subgroup analysis of study type and population characteristics revealed the same results.

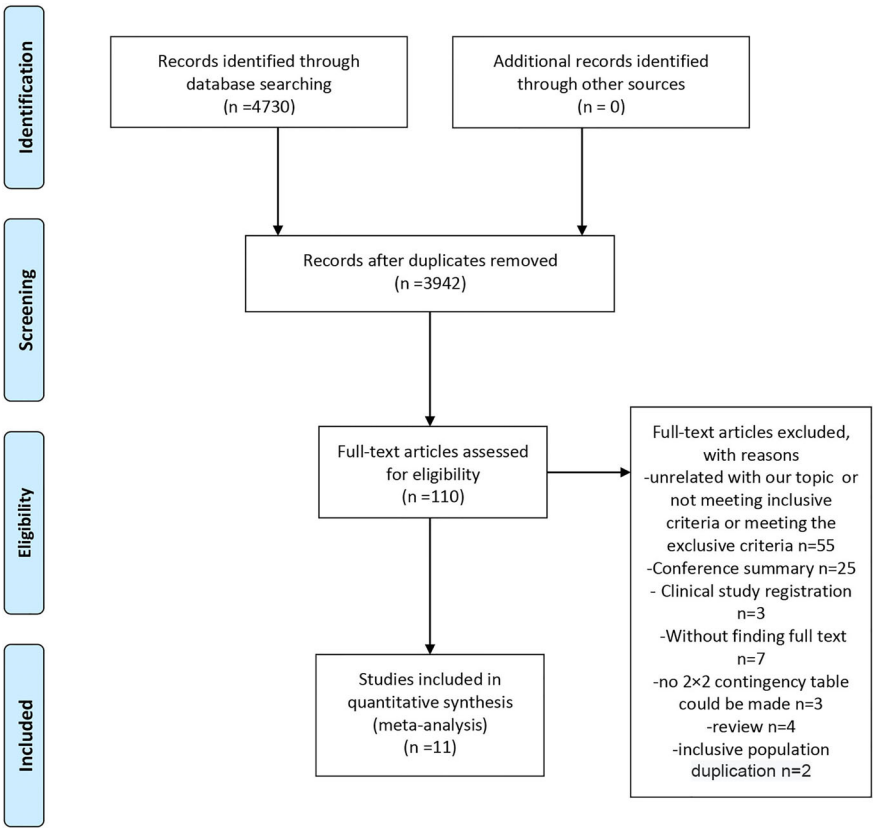

FIGURE 1 | Flow of studies through the review.

TABLE 1 | Characteristics of the studies included in the meta-analysis.

Study	Year	Study type	Population		AMH test		Outcome			
			Population characteristics	Age (y)	Threshold (ng/ml)	Assay	TP	FP	FN	TN
Korsholm	2018	Prospective cohort	–	No limitation	1.3	Elecsy	3	29	25	139
Casadei	2013	Prospective cohort	Unexplained infertility	No limitation	0.75*	IBC	22	5	47	9
Somigliana	2015	Prospective nested case-control study	–	No limitation	1.1*	GEN II ELISA	11	15	65	60
Murugappan	2019	Retrospective cohort	–	No limitation	1	NA	19	28	33	75
Casadei	2018	Prospective study	Ovary cyst	18–45	1.1	GENII ELISA	7	5	7	8
Steiner	2017	Prospective cohort	–	30–34	0.7	Ultrasensitive AMH ELISA	10	22	143	335
				35–44			21	31	85	90
Hagen	2012	Prospective cohort	–	<35	1.82*	GENI ELISA	16	20	60	90
Hvidman	2016	Case-control study: a prospective cohort study with a prospective cross-sectional study	–	<35	0.70*	GENI ELISA	8	6	235	232
				35–40			10	12	129	100
Khan	2019	cross-sectional cohort	–	≤35	0.7	Elecsy	31	16	269	250
				36-39			25	18	98	104
Zhou	2019	Prospective cohort	Endometriosis	≤35	2	IBC	30	12	30	31
Zarek	2015	Prospective cohort	–	18–34	1	GEN II ELISA	22	54	346	640
				35–40			20	28	34	58

**Converted to ng/ml using the conversion formula ng/ml 7.14 pmol/L.*

TABLE 2 | Characteristics of the studies included in the meta-analysis.

Study	Inclusion criteria	Exclusion criteria
Korsholm	(i) Women of reproductive age in a heterosexual relationship who had (ii) tried to conceive naturally or had an unplanned natural conception within 2 years after inclusion. All women included had (iii) a known duration of the pregnancy attempt, and (iv) AMH analyzed by the Elecsys® method	Hormonal contraceptive use at inclusion
Casadei	(1) Unexplained infertility, that is the lack of pregnancy after 1 year of unprotected sexual intercourse in women without apparent disorder of fertility; (2) normal or low ovarian reserve; (3) both ovaries present; (4) regular menstrual cycles	(1) PCOS according to the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004); (2) congenital adrenal hyperplasia; (3) androgen secreting tumors; (4) Cushing syndrome; (5) male infertility; (6) tubal pathologies; (7) anovulation; (8) hyperprolactinemia; (9) hypothalamic amenorrhea; (10) previous ovarian surgery; (11) ovarian tumors; (12) anatomical abnormalities of the uterine cavity; (13) intraperitoneal adhesions; (14) endometriosis and other pelvic pathologies; (15) thyroid dysfunction and other endocrinological disorders such as diabetes mellitus; (16) recurrent pregnancy loss; (17) autoimmune diseases
Somigliana	Inclusion criteria for both cases and controls were: (i) age >18 years, (ii) natural conception (women conceiving with the use of controlled ovarian hyperstimulation with or without assisted reproductive techniques were excluded), and (iii) regular menstrual cycles (24–35 days). Controls were the subsequently referred women matched to cases on the basis of age (6 months, ratio 1:1).	-
Murugappan	Patients with a history of at least two prior pregnancy losses, defined as loss of pregnancy from conception through 20 weeks gestational age, were included.	-
Casadei	Age between 18 and 45 years; ultrasound diagnosis of uni- or bilateral ovarian cysts; absence of malignancy criteria by ultrasound; and absence of endocrine disorders such as thyroid dysfunction, hyperprolactinemia, or Cushing syndrome	Histologic diagnosis of malignancy and perform bilateral ovariectomy; previous adnexal and uterine surgery or chemotherapy; and premature ovarian failure (POF)
Steiner	Women between 30 and 44 years of age had been attempting to conceive for 3 months or less and were cohabitating with a male partner	Fertility problems (history of sterilization, diagnosis of polycystic ovarian syndrome, previous or current use of fertility treatments, known tubal blockage, surgically diagnosed endometriosis) or a partner with a history of infertility. Women who were currently breastfeeding or had used injectable hormonal contraception in the preceding year were also excluded
Zarek	Women in this cohort were attempting pregnancy; were aged 18–40 years, with regular menstrual cycles of 21–42 days in length; and had a history of one to two prior pregnancy losses	history of infertility, pelvic inflammatory disease, tubal occlusion, endometriosis, anovulation, uterine abnormality, or polycystic ovarian syndrome
Hagen	20–35 years old, lived with a partner, and had no children. Couples with no previous reproductive experience who intended to discontinue contraception to become pregnant were eligible for enrolment	-
Hvidman	Study group: infertile patients referred for fertility treatment at The Fertility Clinic, Rigshospitalet, at Copenhagen University Hospital from September 2011 to October 2013. From September 2011, the Fertility Clinic offered newly referred infertile patients an assessment of ovarian and endocrine parameters prior to the first treatment cycle. Patients identified as eligible for the present study were examined on Cycle Days (CD) 2–5 and interviewed to obtain relevant background information. Control group: non-users of hormonal contraception with no history of infertility recruited in a prospective cross-sectional study conducted	Study group: The following patients were considered non-eligible: (i) patients referred for preimplantation genetic diagnosis, (ii) patients referred due to HIV or contagious hepatitis B or C infection, and (iii) single and homosexual women, as they were per se not considered infertile. Furthermore, patients referred directly for oocyte donation (OD) from other fertility centers were not examined on CD 2–5 and thus not included as they had already been diagnosed with a diminished ovarian reserve and most had started hormone replacement therapy or treatment with estradiol to prepare for the OD. Control group: polycystic ovary syndrome (PCOS) defined as oligo- or amenorrhea in addition to AFC ≥12 and/or an ovarian volume.10 ml3 in at least one ovary in accordance with the Rotterdam Criteria

(Continued)

TABLE 2 | Continued

Study	Inclusion criteria	Exclusion criteria
	at the Fertility Clinic, Rigshospitalet, from August 2008 to February 2010.	
Khan	i) no history of gynecological and abdominal surgery, ii) having the normal sonographic texture of ovaries, and iii) with no signs of hyperandrogenemia.	i) Those having any communicable disease or metabolic syndrome, ii) patients referred for pre-implantation genetic testing, iii) patients with polycystic ovarian syndrome (PCOS) and oligo-amenorrhea, iv) patients using any contraceptives, v) those having iatrogenic and autoimmune conditions, vi) obese infertile patients over the age of 40.
Zhou	Patients with an age of 20 to 35 years and a plan to conceive after surgery	Any suspicious findings of malignant disease, recurrent endometriosis, and hormone therapy within 3 months before surgery

DISCUSSION

Our meta-analysis indicated a weak predictive value of AMH for spontaneous pregnancy. The heterogeneity analysis confirmed the robustness of the calculated results. Calculations performed after the age stratification did not show an increased predictive ability.

AMH directly reflects the original follicular pool, and its secretion is unaffected by FSH, possessing the advantages of sensitivity and reliability. AMH has gradually replaced basic FSH as the most reliable ovarian reserve biomarker (3). Despite being one of the most widely used ovarian reserve tests in the clinical setting, a uniformly accepted low AMH cutoff is still lacking (3, 32), and the current primary reference is the Bologna Standard of AMH < 0.5–1.1 ng/ml.

In this meta-analysis, the included studies chose different cutoff AMH values detected by different AMH assays of 0.75–2 ng/ml. As a result, a negative correlation between sensitivity and specificity (known as the threshold effect) was presented. To prevent the benefits of the experimentation from being exaggerated, we plotted the SROC curve. The predictive value was limited when the AUC was 0.5–0.7, better when it was 0.7–0.9, and best when it was >0.9. The overall AUC was 0.5932 and the Q index was 0.5702, suggesting a weak predictive ability.

A woman's serum AMH concentration usually peaks at around 20–25 years of age and gradually decreases with age to undetectable levels (33). The age-related ovarian function decline is generally accompanied by menstrual cycle disorders, ovulation disorders, and oocyte quality reduction, which account for approximately 10% of female infertility cases (34). In addition, decreased estrogen levels also adversely impact endometrial receptivity, the pelvic microenvironment, and other factors, which also leads to a decline in female fertility (34). To fully explore the predictive value of AMH for natural fertility, participants were stratified into older or younger than 35 years of age subgroups. Four studies divided the included participation by age (16, 28, 30, 31), while two studies included only women under 35 years of age (11, 14). An increased AUC and Q index were found in women younger than 35 years of age (0.6355 and 0.6025, respectively). Considering that the AUC was still lower than 0.7, AMH has weak predictive value for spontaneous pregnancy in young women. In women older than 35 years of age, the AUC was 0.5536 and the Q index was 0.5403, indicating poor predictive value.

Our age-stratified results indicated that low serum AMH levels do not necessarily represent decreased natural fertility in younger or older women. Statistics on age-specific AMH levels in Korean women with regular menstrual cycles show that women older than 35 years of age have an AMH level lower than 1.5 ng/ml, a finding that is consistent with the cutoff values that most included studies chose (0.75–2 ng/ml) (35). In younger women, an early decrease in AMH levels suggests an abnormally declined ovarian reserve, which might lead to decreased fertility. However, our meta-analysis showed that the predictive value of AMH for spontaneous pregnancy in this group was limited. AMH levels may be more closely associated with follicle quantity than oocyte quality in young women, which is inferred in some IVF-related studies as well (36–38).

Compared to ovarian reserve, regular ovulation and oocyte quality may hold greater significance in spontaneous pregnancy. Our meta-analysis did not further stratify AMH levels into low *versus* extremely low AMH because of the limited number of original studies. Some researchers believe that active treatment could be considered for young women with extremely low AMH levels, except for those with low AMH levels but no infertility factors (39, 40). Unfortunately, few original studies further divided AMH levels into low and extremely low subgroups; thus, we failed to determine whether there is an improved predictive value. In women of later reproductive age, a significant reduction in ovarian reserve is part of the biological progress due to the accelerated depletion of the follicular pool. Thus, the serum AMH concentration varied from low to undetectable. In this group, a relatively higher AMH concentration might enable a slower follicular failure rate and better conception ability.

A previous meta-analysis synthesized the effect of AMH on implantation, clinical pregnancy, and live birth in IVF, and its results demonstrated that the predictive effect was weak overall, although better but still low in women with DOR. Most studies defined DOR as age >35 years (41, 42). Contrary to the expected outcome, this meta-analysis does not determine a reliable predictive value in women aged >35 years. A decreased AMH level does not indicate decrease natural fertility either. A possible reason for this finding might be that only the oocyte quantity experiences a slower depletion.

A committee opinion on ovarian reserve tests indicated that a suitable crowd should be fully considered. As a screening test, AMH would be more applicable to the general IVF population as well as women at a high risk of DOR than women at a lower risk

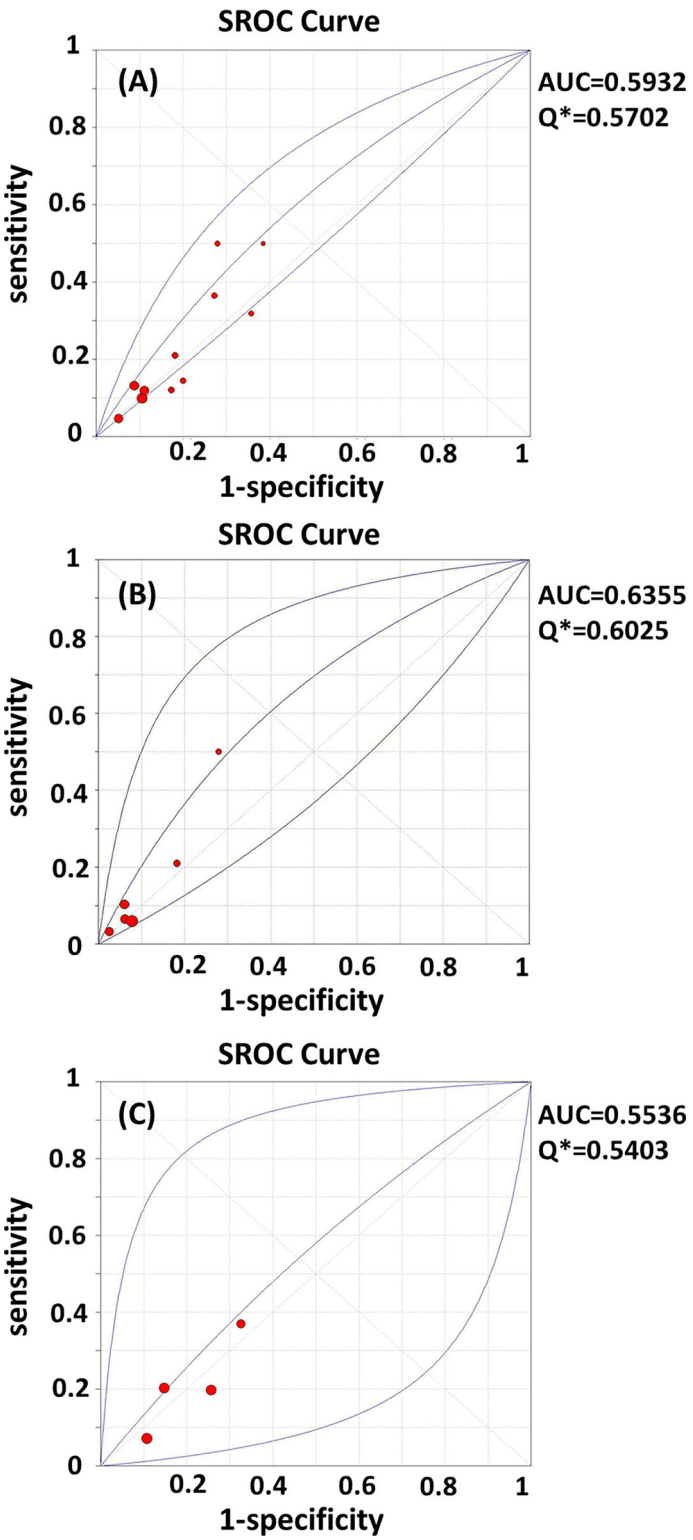

FIGURE 2 | **(A–C)** SROC curves of AMH in the prediction of spontaneous clinical in **(A)** all women; **(B)** women younger than 35 years; **(C)** women elder than 35 years. AUC, area under the summary receiver operating characteristic curve; Q*,Cochran's Q index.

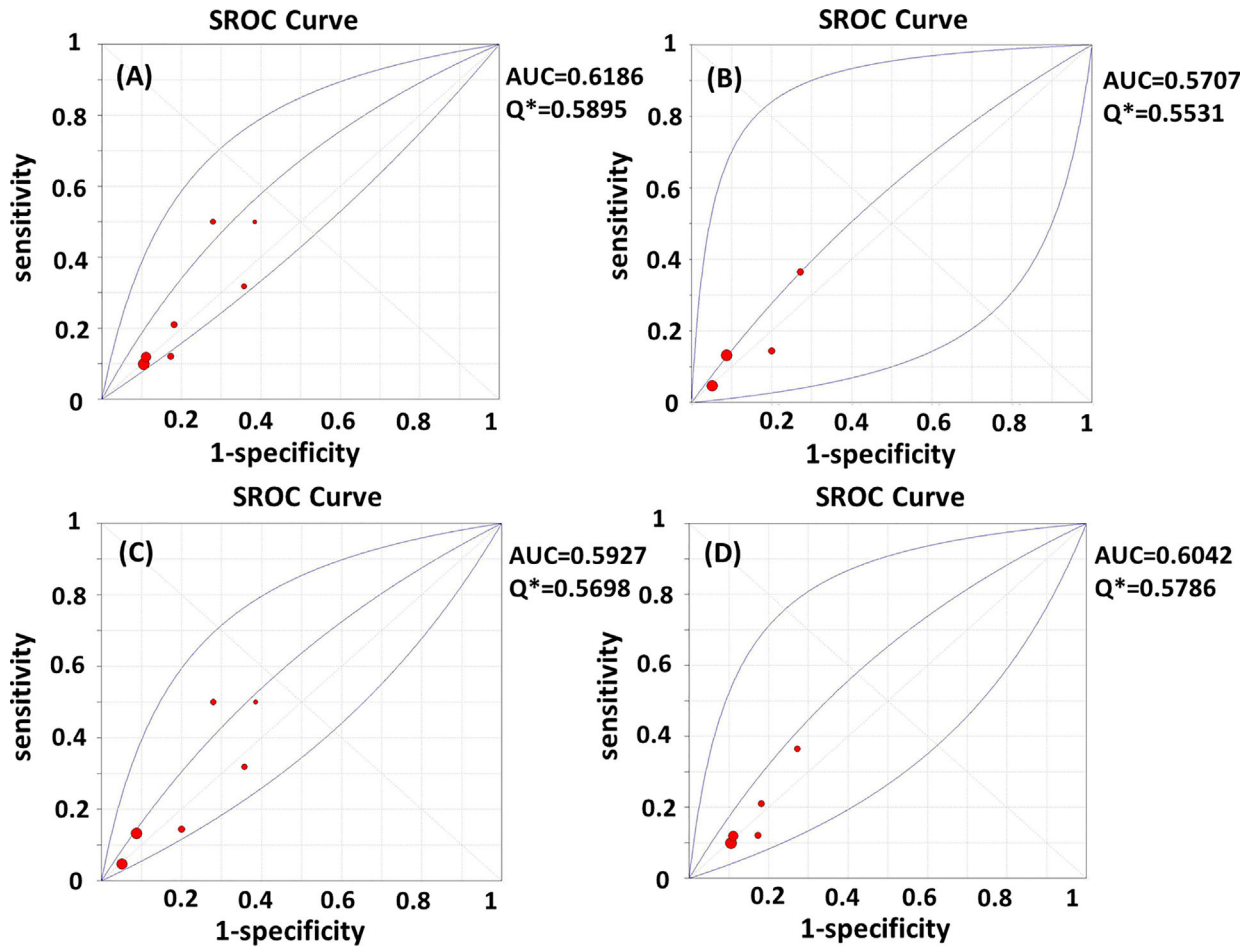

FIGURE 3 | **(A–D)** SROC curves of AMH in the prediction of spontaneous clinical in **(A)** prospective subgroup; **(B)** non-prospective subgroup; **(C)** subgroup with risk factors for infertility; **(D)** subgroup without risk factors for infertility. AUC, area under the summary receiver operating characteristic curve; Q*, Cochran's Q index.

of DOR (3). However, AMH is more universally applied in clinical practice. Therefore, a confirmed quantitative or qualitative relationship between AMH and natural pregnancy is necessary for clinicians to provide individualized fertility guidance. The findings of our meta-analysis might be complementary to previous opinions about natural pregnancy. Meanwhile, it is important to avoid unnecessary fertility anxiety among reproductive-aged females, especially young nulliparas with decreased serum AMH levels.

To our knowledge, this meta-analysis is the first to examine the effects of AMH for natural pregnancy prediction. Furthermore, we also analyzed the effects in young *versus* old subpopulations. However, this study has several limitations. First, most primary studies analyzed AMH levels as predictors of reproductive outcomes using a certain cutoff value. However, in clinical practice, the use of only one cutoff value probably does not reflect the biological situation. We considered the factor of age; however, in each age range, further stratification of AMH values may show increasing predictive value. Unfortunately, studies dichotomized the data using a certain cutoff value, resulting in a non-differential classification error. Second, not all studies were performed in an age-stratified manner. The limited number of subgroup studies might affect the accuracy of the SROC curve. In addition, the included studies were mainly from western countries, which might restrict the application of their findings to other races. Additionally, different AMH assays were performed. Incomparable values and measurement deviations may influence the conclusions. Considering that our meta-analysis only assessed the predictive value of AMH for natural pregnancy, its predictive value for other important reproductive outcomes, such as live birth, requires further exploration.

In conclusion, the findings of existing studies vary regarding whether AMH levels can predict natural pregnancy, and our meta-analysis suggested weak predictive value of serum AMH level for natural clinical pregnancy. A decreased AMH level does not represent decreased natural fertility in young or old females. Thus, caution should be exercised regarding the appropriate application of AMH

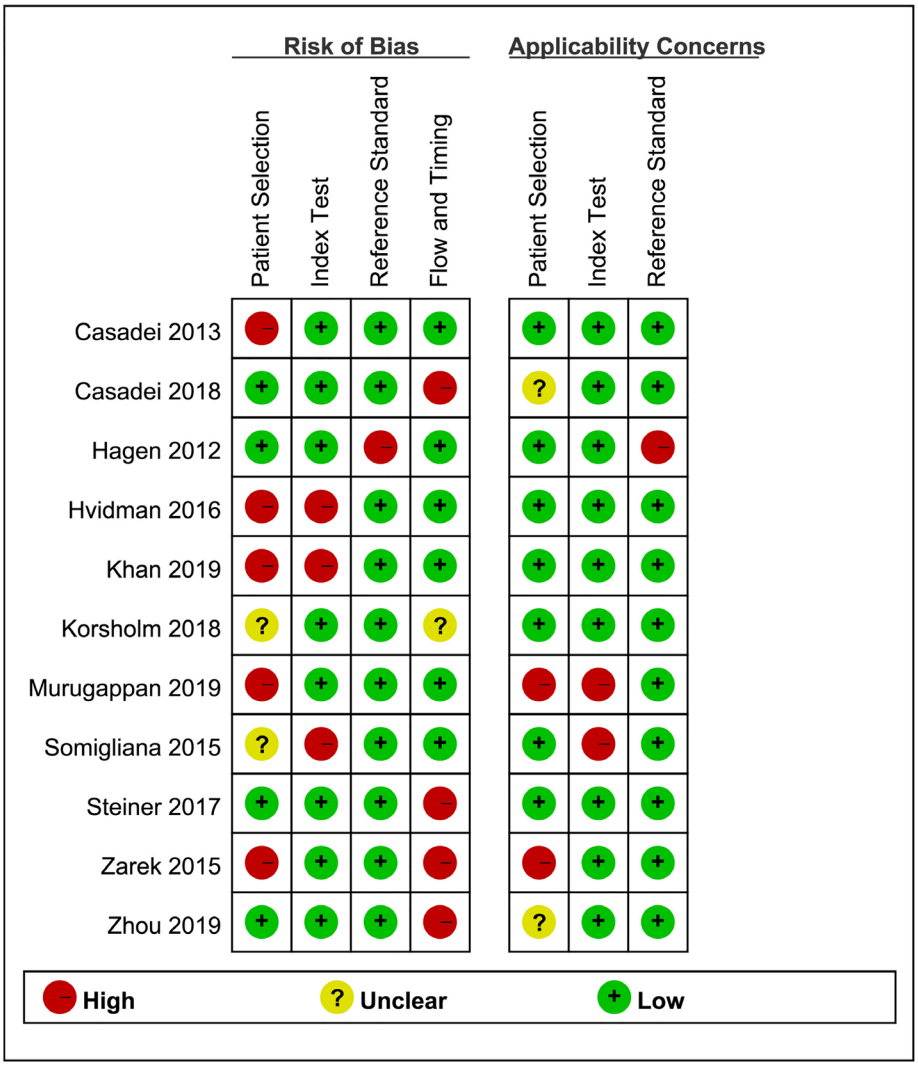

FIGURE 4 | Risk of bias and clinical applicability of the included studies.

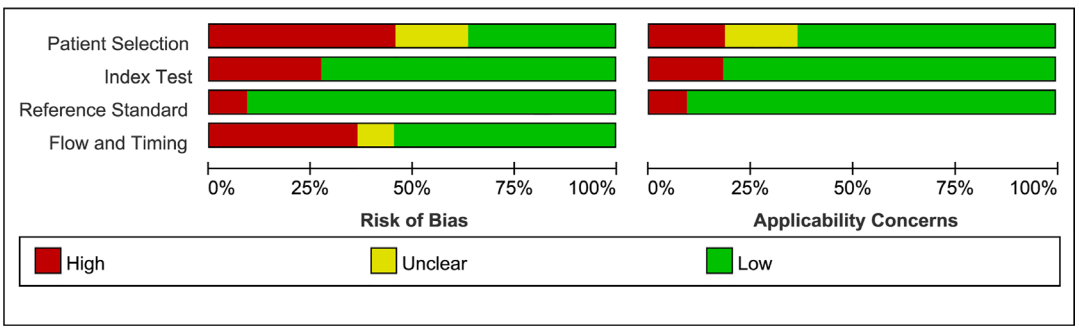

FIGURE 5 | Graphical display for QUADAS-2 results.

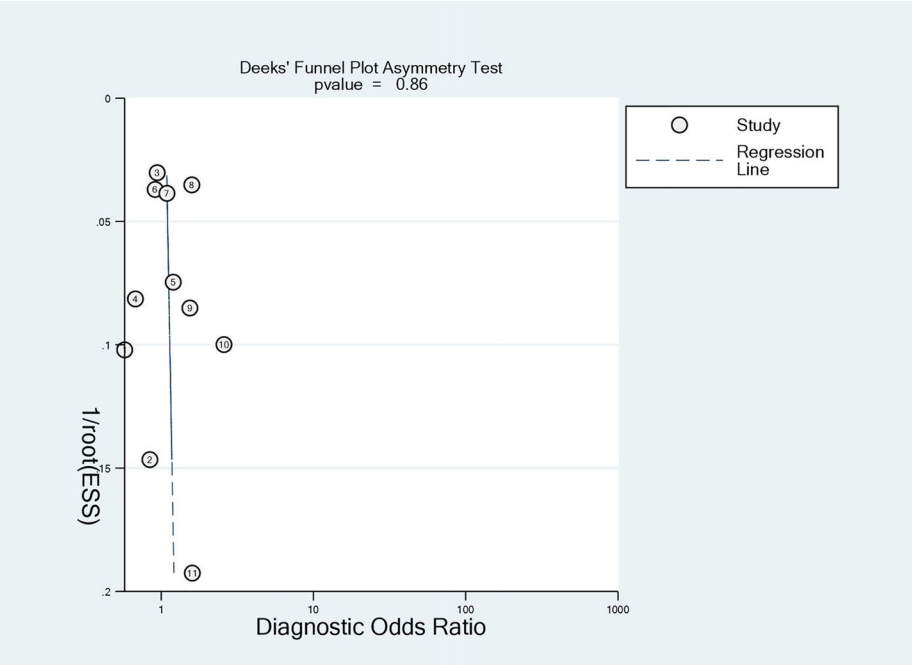

FIGURE 6 | Deeks funnel for publication of included studies.

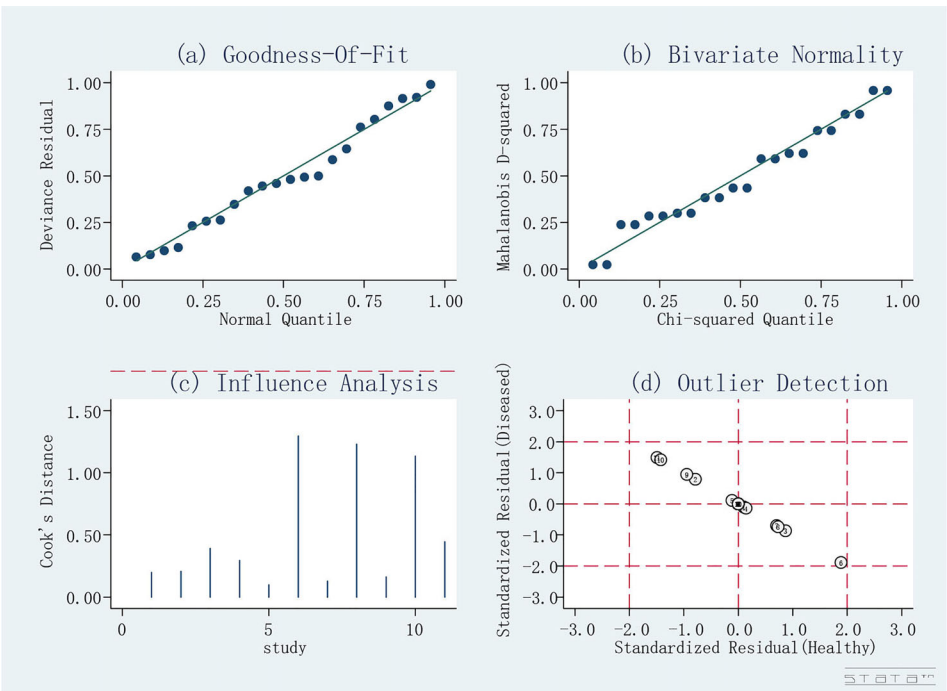

FIGURE 7 | Sensitivity analysis.

measurements, especially in pre-conception counseling, to avoid over-interpreted and unnecessary fertility anxiety.

AUTHOR CONTRIBUTIONS

CL performed the literature screening, the data extraction, the analyses, and the drafting of the manuscript. MJ performed the literature screening, data extraction. CL and MJ have contributed equally to this work. WZ reviewed the protocol. XT and QC drafted the protocol. XW and YZ extracted the baseline characteristic. RZ contributed to the literature inclusion and revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Clinical Application of AMH Measurement in Assisted Reproduction

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Anti-Müllerian hormone reflects the continuum of the functional ovarian reserve, and as such can predict ovarian response to gonadotropin stimulation and be used to individualize treatment pathways to improve efficacy and safety. However, consistent with other biomarkers and age-based prediction models it has limited ability to predict live birth and should not be used to refuse treatment, but rather to inform counselling and shared decision making. The use of absolute clinical thresholds to stratify patient phenotypes, assess discordance and individualize treatment protocols in non-validated algorithms combined with the lack of standardization of assays may result in inappropriate classification and sub-optimal clinical decision making. We propose that holistic baseline phenotyping, incorporating antral follicle count and other patient characteristics is critical. Treatment decisions driven by validated algorithms that use ovarian reserve biomarkers as continuous measures, reducing the risk of misclassification, are likely to improve overall outcomes for our patients.

Keywords: anti-Müllerian hormone, assisted reproduction, *in vitro* fertilization, intrauterine insemination, ovarian response, gonadotropin dosing

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INTRODUCTION

One major clinical area where anti-Müllerian hormone (AMH) measurement has carved out a clear niche is in assisted reproduction. Over the recent two decades, there have been a growing number of observational studies and randomized controlled trials (RCTs) exploring AMH's role in assessing ovarian reserve, predicting ovarian response and outcomes to treatment, and clarifying its utility for individualizing treatment strategies in women undergoing assisted reproduction treatment (ART) including intrauterine insemination (IUI) with controlled ovarian stimulation or *in vitro* fertilization (IVF). These clinical applications are based on AMH exhibiting strong correlations with the primordial follicle count (1), the ultimate parameter that represents the conceptual ovarian reserve, as well as the later stages of follicular development that are responsive to gonadotropins and constitute the functional ovarian reserve (2–4). The widespread clinical adoption of AMH has been further enhanced through the ease of scalability of serum/plasma testing, the availability of high precision automated assays, that it can be measured at any part of the menstrual cycle, that small fluctuations observed within and across cycles have been shown not to be clinically important (5–11), and that measurements determined in the months leading up to the index stimulation cycle can

accurately guide assisted reproduction treatment decisions. In this review, we summarize the recent evidence underpinning the use of AMH in ART, including how AMH can inform the overall prognostic phenotype and individualize treatment decisions, while highlighting the areas that continue to require further exploration.

INITIAL OBSERVATIONS

AMH as a Continuous Measure

The AMH concentration that we receive from that initial blood test is like many biological measures lying on a continuum, from very low at one extreme to very high at the other. However, historically studies have primarily focused on identifying individual threshold values to categorize different types of prognosis and stratify treatments (12, 13). In clinical practice it is often helpful to label individuals as having or not having an attribute, such as a potential poor responder, depending on the value of a continuous variable like AMH. However, the dichotomization of continuous variables leads to several problems (14). Importantly much information is lost, so the statistical power and ability to demonstrate an association with the outcome is substantially reduced. Secondly the extent of the variation in outcome between groups may be underestimated, such that individuals close to but on opposite sides of the threshold are characterized as being very different rather than being very similar. Thirdly, using two groups conceals any non-linearity in the relation between the variable and outcome. Lastly, the use of apparently "optimal" cutpoint (usually that giving the minimum P value) runs a high risk of a spuriously significant result; the difference in the outcome variable between the groups will be overestimated, perhaps considerably, and the confidence interval will be too narrow. In ART these issues are not unique to AMH and apply to many routine indices like antral follicle count (AFC), sperm counts, and endometrial thickness which are all continuous measures yet we continue to dichotomize them rather than treat them as continuous variables to enable greater variability in outcomes to be explained. Confirmation of the value of treating AMH as a continuous measure, enabling greater explanation of variability, has recently been shown in a RCT (15). Simple categorization has a role, but as we become more sophisticated in our understanding of the strengths and weaknesses of AMH we propose that we should consider AMH as biology intended—a continuous measure of the functional ovarian reserve.

Ovarian Reserve Markers Should Not Be on the Causal Pathway When Estimating Strength of Associations With Outcomes

Assessment of the true strength of the correlation between an exposure (such as AMH) and an outcome (for example oocyte yield) requires that the exposure in no way influences the treatment pathway which results in the outcome. Unfortunately for many of the studies that blindly evaluated the correlation of AMH with outcomes (16, 17), also measured and acted upon the antral follicle count (AFC), which is itself strongly correlated with AMH due to the granulosa cells of the smaller antral follicles being the primary source of AMH (4). By altering the stimulation strategy or dose based on the AFC or other linked factors like age, the researchers will have introduced systematic bias which can lead to an overestimation of the strength of the correlation, which will apply to the primary marker used such as AFC and to a lesser degree the inter-related markers like AMH. The only way the strengths of the association can be truly evaluated is if all patients are treated identically and the researchers are blind to the initial ovarian reserve biomarkers (18). Such a study design is often only seen in randomized controlled trials (19, 20), rather than observational studies which are frequently used to assess and report the relative merits of different biomarkers. It is in this context of multicenter large scale RCTs with strictly defined protocols, where a true indication of the performance and limitations of biomarkers such as AFC and AMH can be observed.

ROLE OF AMH MEASUREMENT IN IUI WITH CONTROLLED OVARIAN STIMULATION

Controlled ovarian stimulation and IUI can be a first-line treatment for unexplained infertility as well as infertility due to mild male factor or endometriosis. In this context IUI may commonly be coupled with controlled ovarian stimulation by gonadotropin or oral anti-estrogen drugs which may correct subtle problems of ovulation, slightly increase the number of oocytes available for fertilization, and enhance the accuracy of timing of insemination (21, 22).

For AMH to be of value in this context, we anticipate it would identify which patients would benefit from stimulated IUI rather than proceeding directly to IVF, identify an appropriate initial stimulation strategy for example exogenous gonadotropins or aromatase inhibitors, and/or identify the likely prognosis to manage patient expectations regarding the likelihood of success. Unfortunately, although AMH contributing to all three aspects may be aspirational there have been a limited number of studies evaluating the role of AMH in IUI management. Historically, studies focused on the association of pretreatment AFC with pregnancy outcomes, with differing conclusions (23–25). The first study on the role of serum AMH in predicting treatment outcome after ovarian stimulation using gonadotropins was reported in 2010 in 243 women undergoing IUI (26). In this study, baseline AFC was used to alter the dose of starting gonadotropin (ovarian stimulation was achieved with hMG or recombinant FSH starting at 150 IU/day, except for those with AFC ≥10 or polycystic ovaries, where it was started at 100 and 75 IU/day, respectively). In women who attained live birth either in the first treatment cycle or cumulatively over three treatment cycles, their pretreatment AMH was significantly higher than those who did not (median AMH 3.47 ng/ml vs. 2.04 ng/ml). Furthermore, AMH remained a significant predictor on the likelihood of cumulative live birth after controlling for age and body mass

index of the women in a logistic regression model. Others have subsequently reported similar associations between higher AMH and higher success rates (27–29). To date validation of optimal treatment strategies (30) or of retrospectively derived algorithms (31) have however been limited and would be the next step for confirmation of a more widespread role of AMH in stimulated IUI programs.

ROLE OF AMH MEASUREMENT IN IVF

In IVF program, numerous studies have explored the role of serum AMH measurement in predicting ovarian response to gonadotropin stimulation, individualizing treatment pathways to improve efficacy and safety, and lastly predicting overall treatment success (12, 13, 18, 32). Given the biological premise of AMH as a functional ovarian reserve marker, it is not surprising that it is these first two areas where AMH has made the greatest contribution.

Prediction of Suboptimal or Excessive Ovarian Response

Ovarian stimulation forms an integral part in modern IVF programs. Multiple follicle development and aspiration, and hence collection of multiple oocytes, helps to increase the efficiency of the treatment program. It has been reported that a higher oocyte yield up to around 15 was associated with higher live birth rate in the fresh treatment cycle (33) as well as higher cumulative live birth rate following the fresh and all frozen-thawed embryo transfers after one IVF cycle (34).

It is now widely established that AMH and AFC are the currently best available predictors of ovarian response and its associated extremes; poor and excessive ovarian response (35–37). Accepting the limitations noted above on using observational studies, an individual patient data (IPD) meta-analysis assessing prediction of excessive ovarian response included 57 studies with 4,786 women and concluded that both AMH and AFC exhibited similar and reasonably good performance in predicting excessive ovarian response in isolation. The area under the receiver operating characteristic (ROC) curve for AMH and AFC was 0.81 and 0.79 respectively (16). Although the combination improved the area under the curve marginally to 0.85, inclusion of additional covariates such as age or FSH did not improve the prediction further. A second IPD meta-analysis reported on the prediction of poor ovarian response (17). It included data from 28 studies with 5,705 women undergoing IVF treatment. Again, both AMH and AFC had similar and reasonably good performance in predicting poor ovarian response on their own, with an area under the ROC curve of 0.78 and 0.76 respectively, and once again combining the two or adding age did not significantly improve the prediction. However, as noted for almost all of the studies included in these meta-analyses, AFC was known prior to commencing ovarian stimulation and used to modify the dose which might have led to an overestimation of the strength of the association of AFC.

In the context of predicting suboptimal ovarian response, AMH and AFC were among the criteria used to define or predict poor ovarian responders in the Bologna criteria (38) and more recently the Poseidon classification (39). Although the Poseidon criteria has been proposed as an attempt at defining a more homogeneous population, heterogeneity remains. For example, the thresholds for AMH and AFC are not aligned with respect to established correlations between these two indices (4). For treated patients there is no agreed consensus on the nature of the previous stimulation strategy. Lastly patients are dichotomized to either <35 or ≥35 years of age, despite the non-linear relationship with oocyte aneuploidy.

An extrapolation of the ability to predict ovarian response is the individualization of the ovarian stimulation regimen in the treatment naïve patient, particularly if aiming for a fresh embryo transfer. In this context achieving an optimal oocyte yield while minimizing the risk of ovarian hyperstimulation syndrome (OHSS) is paramount. Our initial suggestion of AMH-driven algorithms to determine the initial gonadotropin dose and regimen of ovarian stimulation (40, 41) have now been confirmed in several RCTs, with the largest (n=1326) assessing the efficacy and safety of follitropin delta (15). In this RCT, the follitropin delta dose was based on the individual women's serum AMH level and body weight and compared with follitropin alpha at 150 IU with subsequent step up or down according to ovarian response. The two treatment arms had similar mean oocyte yield and live birth rates, and yet the follitropin delta arm had significantly lower rates of suboptimal or excessive response. A recent Cochrane meta-analysis concluded that although individualized dosing of gonadotropin based on ovarian reserve markers might not influence the rate of ongoing pregnancy or live birth compared to standardized dosing, it could reduce the incidence of moderate-to-severe OHSS by prompting the use of a reduced dose of gonadotropin in predicted high responders (42).

As current AMH assays give different numerical results without any universal standardization (43, 44), and that reported studies were performed using different assays, it is not possible to combine the available data to determine cut-offs for predicting excessive or suboptimal ovarian response. Similarly, although historical definitions including the Bologna criteria defined poor ovarian reserve as an AFC of below 5 to 7, or AMH level of below 0.5–1.1 ng/ml, while the Poseidon classification adopted an AFC of 5 or AMH of 1.2 ng/ml as the cut-offs for defining it, it should be noted that these were based on previous studies using different assay methods and hence there may be problems to adopt these apparently simple thresholds universally.

Prediction of Pregnancy or Live Birth in IVF Treatment

Despite the good performance of AMH and AFC in predicting ovarian response, most studies, however, consistently showed that just like age, AMH and AFC were poor overall predictors of pregnancy or live birth in the fresh IVF cycle (17, 32, 45). The summary ROC curves derived from the individual patient data meta-analysis by Broer et al. (17) for prediction of ongoing pregnancy confirmed the limited role of AMH, AFC and age, or

their combinations, with an area under the ROC curve of less than 0.6. Focusing on AMH and live-birth, a meta-analysis on 13 studies found an area under the ROC curve of 0.61 (95% confidence interval 0.56–0.65) of confirming the limited contribution that AMH in isolation would have for prognostication of overall live-birth (32).

All these studies were limited by focusing on the rate of pregnancy or live birth in the fresh IVF cycle only. In modern-day IVF programs, embryo cryopreservation constitutes an increasingly important part, and hence the cumulative live birth rate from the fresh and all frozen-thawed embryo transfer (FET) cycles combined would be more informative and meaningful than the outcome of the fresh cycle alone (46). A retrospective analysis evaluating the role of baseline AMH in predicting cumulative live birth from the fresh IVF cycle plus all subsequent FET cycles derived from that stimulated cycle was first reported in 2013 (3). It included 1,156 women undergoing the first IVF cycle in a single center treated under the long GnRH agonist protocol or GnRH antagonist protocol. It suggested that the cumulative live birth rate followed a gradual rising trend with serial increase in serum AMH or AFC over a continuum instead of showing an abrupt change at any threshold value. However, both parameters had only modest performance, which was not better than the women's age alone, in predicting the absolute occurrence of cumulative live birth as demonstrated by the ROC curves (area under the curve being 0.646, 95% CI 0.616–0.675). After controlling for the women's age and the number of embryos replaced, both serum AMH and AFC were not significant independent predictors of live birth in the fresh IVF cycle nor cumulative live birth suggesting that their association with overall livebirth was through the number of oocytes and thereby number of embryos available to transfer. Another recent study in 9,494 Chinese women similarly demonstrated that increasing AMH up to 5–7 ng/ml predicted better cumulative live birth rate in IVF and that it was mainly through the association with oocyte yield (47).

As for women with predicted poor ovarian reserve, a recent retrospective analysis on 825 IVF cycles showed that the live birth rate decreased through Poseidon groups 1, 3, 2, and 4 in order (48). It implies that both AMH or AFC as well as age have an impact on the prediction of live birth. It is worth to note that in the study by Li et al. (3), women with serum AMH <0.5 ng/ml still had a cumulative live birth rate of 27%, and cumulative live birth did occur in women with AMH as low as 0.15 ng/ml. Another secondary analysis on women in the OPTIMIST study, a prospective observational study on 551 women with predicted low prognosis, showed that those in Poseidon 4 group (older women with low ovarian reserve) still had conservative and optimistic cumulative live birth rates of 37% and 41% respectively over 18 months of treatment (49).

Collectively all of these data suggest that a patient at any age with a higher AMH has an overall better prognosis. However, due to the limitations of its predictive performance, a threshold value should not be used to deny women from attempting ART, nor to be too pessimistic regarding prognosis based solely on an AMH value.

Comparing AMH Versus AFC in Predicting Ovarian Response

Although differences in performance characteristics of AMH and AFC have been reported in several multi-center RCTs (18), direct head-to-head performance comparison of AMH- or AFC-based dosing algorithms has been more limited. Specifically, two RCTs have compared the performance of a serum AMH or AFC algorithm in predicting ovarian response in an IVF program, with both concluding that there were no significant differences in the proportion of cycles attaining desired ovarian response when the gonadotropin dosing algorithm was determined based on either AMH or AFC (50, 51). In the first study, 348 Vietnamese women were treated with a long GnRH agonist protocol, and 35.2% versus 28.4% of cycles attained the desired response when the AMH-based and AFC-based algorithms were adopted respectively ($p>0.05$), although the incidence of hyper-response was significantly lower in the AMH group (8.6%) compared to the AFC group (17.4%) (50). In the second study, 200 participants from Hong Kong were treated on a GnRH antagonist protocol (51). There were no significant differences in the proportion of cycles with desired response between the AMH-based and AFC-based groups (49.0% versus 54.0%, $p>0.05$), or the number of oocytes retrieved or the follicular output rate. However, significantly more women required an increase in their gonadotropin dose in the AMH group compared to the AFC group. These findings suggest that clinicians who choose to use these specific published algorithms and treatment strategies would obtain equivalent results whether they use AMH or AFC. However, this conclusion of equivalence does not extend to other untested algorithms or equate to overall equivalence for treatment decision making.

Discordance Between AMH and AFC in Prediction of Ovarian Response

For most women AMH and AFC will be similar, but discordances can occur with extreme disagreements the most concerning and difficult to interpret clinically. A retrospective analysis on 1,046 women assessed the discordance between AMH and AFC, by using the 25^{th} and 75^{th} centiles of AMH (1.4 and 5.3 ng/ml) and AFC (6 and 14) respectively as the thresholds (52). In these analyses only 4 patients exhibited a high AMH but a low AFC and conversely 1 patient exhibited a high AFC and low AMH. Simple categorization may however over emphasize apparent milder discordances, for example in the above study an AMH of 1.3 ng/ml and an AFC of 7 would be recorded as discordant, but clinically many would perceive as equivalent with a similar response anticipated. In the trial by Li et al. (51), among the 200 enrolled women, 26.5% showed discordance between categorization based on AMH or AFC in the pre-treatment cycle ($\kappa=0.560$), with an overall discordance rate of around 30%. In women who were discordant in AMH and AFC categories, those having higher AMH within the same AFC quartile had significantly higher oocyte yield and cumulative live birth rate, and the ovarian responsiveness was intermediate between those where AMH and AFC were concordant on

either the high or low end (52). Applying to clinical scenarios where AMH and AFC categories are discordant, it is reasonable to suggest an intermediate dose of gonadotropin between that assigned for the high and low ends. Nonetheless, such a recommendation will require verification in prospective trials.

In view of such discordant scenarios, it has been proposed that a more holistic phenotype which incorporates AMH, AFC and age can be combined into a composite score for the purpose of ovarian response prediction. The ovarian response prediction index (ORPI), calculated as the product of AMH level (ng/ml) and AFC divided by age of the woman (years), was first reported by Oliveira et al. (53). The original study showed that ORPI had good prediction on oocyte yield, and the same group subsequently also showed that using ORPI for individualization of the ovarian stimulation regimen resulted in elimination of OHSS in their center (54). A retrospective analysis on 285 women stimulated with a standardized initiation dose using corifollitropin alpha in the GnRH antagonist protocol confirmed that ORPI was significantly correlated with the oocyte yield (55). ROC curve analysis revealed that the area under the curve for ORPI was comparable to AMH alone and significantly higher than AFC alone for prediction of excessive response, while it was significantly higher than that of AMH or AFC alone for prediction of suboptimal response. In contrast in the phase II derivation of the follitropin delta algorithm, the inclusion of either or all of age, FSH, or AFC did not increase explanation of the variance by ≥5% above what was initially observed for just bodyweight and AMH. Therefore, although a composite index of ovarian reserve biomarkers may be worth further exploration to try to reduce the unexplained variance in ovarian response in future trials, its overall contribution may be limited and would require systematic and timed scanning.

Timing of AMH Assessment Prior to IVF

A number of studies have reported inter-cycle fluctuations of AMH level, and yet the absolute magnitudes of these fluctuations are small and may have limited clinical importance. It was shown that when AMH was measured one month prior to IVF as well as at the start of ovarian stimulation, there was moderate concordance between AMH categorization measured in the pre-treatment versus the stimulation cycle (κ = 0.573) (51). Similarly, an analysis of 1326 women in the three months leading up to an index cycle suggested strong correlations (r=0.92), with no systematic variation across the menstrual cycles (56). Others have also shown using different gonadotropins that it can be used in advance of the index cycle for prediction of response (13, 57). Hence, although the assessment of AMH can be performed on any day of the cycle in the months preceding ovarian stimulation, as for any response prediction the accuracy will be greatest if it is measured in the index cycle immediately prior to commencing gonadotropins.

Prediction of Embryo Quality

There are contrasting data reported on the role of AMH in predicting embryo quality. While some studies revealed that serum AMH was not significantly associated with morphokinetic embryo quality as assessed by time-lapse imaging (58, 59), there were reports that the oocyte-specific AMH concentration in follicular fluid had good prediction on embryonic development and live birth (60, 61). It is interesting to further explore the functional relationship of follicular and serum AMH with oocyte competence, embryo euploidy and its role in embryo selection.

CONCLUDING REMARKS

AMH has evolved as a useful tool for the assessment of the functional ovarian reserve and prediction of ovarian response, with performance at least equivalent to or better than AFC. However, just like AFC or age, its ability to predict live birth both in the fresh cycle and cumulatively taking into account all embryos derived from the same index stimulation cycle is limited, and primarily stem from its relationship with the oocyte and hence embryo yield. We propose that we no longer need to debate on which biomarker is best, but rather accept that we can utilize all of the information at our disposal to characterize the baseline phenotype and likely response and modify our treatment strategies accordingly. The use of both AMH and AFC, as continuous measures, combined with other patient characteristics in validated algorithms will reduce the risk of misclassification and is likely to improve overall outcomes for our patients.

AUTHOR CONTRIBUTIONS

HL and SN conceived, wrote, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Clinical Value and Interpretation of Anti-Müllerian Hormone in Women With Cancer

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Cancer treatments can be damaging to the ovary, with implications for future fertility and reproductive lifespan. There is therefore a need for a biomarker than can usefully provide an assessment of the ovary and its potential for long-term function after cancer treatment, and ideally also be of value pre-treatment, for the prediction of post-treatment function. In this review we assess the value of anti-Müllerian hormone (AMH) in this context. Measurement of AMH at the time of cancer diagnosis has been shown to be predictive of whether or not there will remain some ovarian function post-treatment in women with breast cancer, in conjunction with age. AMH may however be reduced at the time of diagnosis in some conditions, including lymphoma, but probably not in women with breast cancer unless they are carriers of *BRCA1* mutations. Following chemotherapy, AMH is often much reduced compared to pretreatment levels, with recovery dependent on the chemotherapy regimen administered, the woman's age, and her pretreatment AMH. Recent data show there may be a long duration of relative stability of AMH levels over 10 to 15 years prior to decline rather than a rapid decline for many young women after cancer. Post-treatment AMH may have utility in determining that ovarian function will not recover, contributing to assessment of the need for ovarian suppression in women with hormone-sensitive breast cancer. AMH measurement provides an index of treatment gonadotoxicity, allowing comparison of different treatment regimens, although extrapolation to effects on fertility requires caution, and there are very limited data regarding the use of AMH to estimate time to menopause in the post-cancer setting.

Keywords: ovarian reserve, cancer, anti-Müllerian hormone, fertility preservation, premature ovarian insufficiency

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INTRODUCTION

Adolescent and young adult cancer survivors can experience many late effects related to their cancer treatment. With substantial improvements in survival following many common cancers, perhaps most importantly in the present context for women with breast cancer (1), and recognition of the importance of quality of life following treatment, identifying, and treating reproductive and sexual health late effects has become increasingly important for these young women. While not all cancer treatments adversely impact the ovary, the overall likelihood of a woman having a pregnancy following cancer treatment is reduced by nearly 40% across all diagnoses (2). Cancer survivors

express considerable unmet informational needs on individualized risks of adverse sexual and reproductive health outcomes and clinical management options, contributing to lower quality of life and distress (3, 4).

Ovarian function, specifically the development of ovarian follicles and associated reproductive hormones, contributes directly to reproductive and sexual health in this population. Measures of ovarian function for diagnostic and predictive indications may help clinicians and patients understand their current as well as predicted future ovarian function. While conventional indicators of ovarian function, namely cyclical menstruation, gonadotropins, estradiol, and progesterone remain essential for assessing current ovarian function (growth of larger follicles and ovulation), the last 15 years have seen a substantial interest in the potential value of measuring anti-Müllerian hormone (AMH). The objective of this review is to discuss current understanding of the clinical value of measuring AMH in young cancer survivors at cancer diagnosis and post-treatment. Specific questions we address include whether AMH levels at cancer diagnosis and/or post-treatment predict response to ovarian stimulation in the short-term, and fertility or time to menopause in the long-term. We also discuss how AMH levels reflect current ovarian function and help to estimate the gonadotoxicity of cancer treatments.

Other reviews in this collection will address many of the details surrounding the origins of AMH and what is known about its role and value in normal ovarian physiology. For the present context, the key points are that AMH is not produced by primordial follicles, but it is produced by the granulosa cells of growing preantral and small antral follicles. There is a relationship between the number of primordial follicles and the number of growing follicles in the adult human ovary (5), and the limited data available show that AMH levels correlate with the number of primordial follicles (6). Thus, AMH is an indirect marker of the true ovarian reserve, i.e., the number of non-renewable, non-growing primordial follicles. Importantly, AMH production by granulosa cells falls dramatically when follicles reach a diameter of approximately 10 mm, and it is estimated that follicles of 5 to 8 mm diameter contributes the majority of circulating AMH (7). These follicles will have been in the growth phase for a significant period of time, probably many weeks, and are approaching a key timepoint when they may or may not be selected for dominance and ovulation, with the great majority, as at all stages of follicle growth, destined for atresia. Thus, AMH may be regarded as a reflection of what can be termed the functional ovarian reserve, which is those follicles which are starting to produce estrogen and in contributing to basal, early follicular, estrogen production, and underpinning the potential for ovulation. This is, of course, the basis for the value of AMH-based treatment strategies in assisted reproduction, as an index of follicular response to ovarian stimulation.

A further important attribute of AMH is that is it detectable in the circulation in childhood as well as in adults, though with complex age-dependent changes (8, 9). Specifically, after a temporary neonatal peak, AMH levels are initially low in childhood, rising to a plateau after puberty until the mid 20s, then progressively decline thereafter to undetectable levels associated with the menopause. It is important to recognize that these physiological changes in AMH levels may result in a peri-pubertal decline and then rise in adolescent girls, which complicates interpretation during puberty and the years thereafter. While the relationship between AMH and the ovarian reserve thus changes between childhood, adolescence and early adulthood, and the main reproductive years (10), the measurement of AMH allows some assessment of ovarian function in prepubertal girls as well as young adults.

MEASURING AMH BEFORE CANCER TREATMENT

The measurement of AMH at the time of diagnosis in women with cancer has two important clinical uses. Typically measured in clinics, most immediately, it is of value in assessing the functional ovarian reserve in women considering ovarian stimulation for fertility for egg or embryo vitrification for fertility preservation, and therefore the question is whether it has the same predictive value as in the normal situation in women having assisted reproduction. Second, in conjunction with age and cancer treatment, it may be of value in predicting long-term ovarian function after cancer treatment is completed.

AMH and Ovarian Stimulation for Fertility Preservation Before Cancer Treatment

Data showing that AMH levels are reduced in women with lymphoma at the time of diagnosis compared to age match controls (11, 12). This may reflect the systemic inflammatory nature of lymphoma compared to other cancers, as AMH levels do not appear to be reduced in women with breast cancer (13, 14). Higher AMH predicts higher oocyte yield in ovarian stimulation of cancer patients (15). Overall, the results of ovarian stimulation with regard to number of oocytes retrieved and proportion fertilized are similar in women with cancer to women without cancer (16). There is, however, evidence of reduced oocyte quality compared to women cryopreserving oocytes for elective purposes (17), which is not reflected by AMH.

AMH in Women With BRCA Mutations

A special situation in the context of breast cancer is the potential impact of mutations in the *BRCA1* and *BRCA2* genes on ovarian function. These genes encode proteins involved in the DNA damage repair pathway, which is of key importance in the oocyte (18), and there is good evidence from animal models that *BRCA1* in particular is necessary for normal fertility and ovarian lifespan (19). That study also suggested that women with *BRCA1* mutations also had lower AMH levels, and a reduced response to ovarian stimulation, and *BRCA1/2* carriage has been linked with an earlier age at natural menopause (20). It appears that women with *BRCA1* mutations, but probably not those with *BRCA2* mutations, do have a lower AMH level overall; it was found to be 25% lower in a study including 172 *BRCA1* mutation

carriers (21), who were also more likely to have AMH levels in the lowest quartile (odds ratio 1.84, 95% CI: 1.11–303). Comparably, a reduced response to ovarian stimulation has also been reported in a cohort of *BRCA1* mutation carriers, with no effect in *BRCA2* mutation carriers (22). Others have found that AMH levels are not reduced in *BRCA* mutation carriers (23, 24), but those studies did not separately analyze *BRCA1* and *BRCA2* carriers.

AMH at Cancer Diagnosis and Prediction of Long-Term Ovarian Function

The value of AMH measurement at the time of diagnosis in predicting long term ovarian function has been clearly demonstrated in women with breast cancer, with limited data for women with other diagnoses. Studies require a long-term prospective cohort recruited at the time of diagnosis, and are therefore relatively few in number. In women with breast cancer, pretreatment AMH predicts long term ovarian function measured as ongoing menses or not. In the first such analysis AMH was shown to have a better predictive value than age (25, 26), although the latter is also, of course, an important predictive factor. In a second similar prospective cohort, it was shown that pretreatment AMH below the median value for the group, 0.46 ng/ml, accurately predicted amenorrhea in all women at 2 years following diagnosis (27). Combining these two cohorts allowed production of a mosaic chart showing the interaction between age and AMH and pretreatment AMH in predicting whether or not the woman was likely to have long term amenorrhea at—2 to 5 years after diagnosis (**Figure 1**). Subsequently larger cohorts followed prospectively from diagnosis within breast cancer treatment trials (28) and specifically recruited have produced confirmatory data (29–31). In survivors of cancers other than breast cancer, it has been shown that pretreatment AMH impacts on the rate of recovery of AMH after chemotherapy, with higher pretreatment AMH associated with more rapid recovery (32). Importantly, the menstrual and AMH outcomes of these studies are limited in part by heterogeneity of definition on duration and timing after treatment, but indicate ovarian function and estrogen production, important to sexual and bone health. To date, however, no studies have investigated how pre-treatment AMH levels are related to post-treatment fertility or time to menopause in women with preserved ovarian function after cancer treatment.

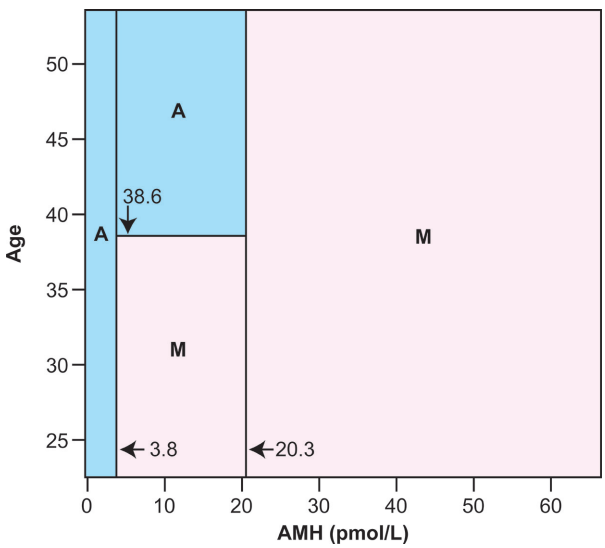

FIGURE 1 | Classification mosaic chart for ongoing menses (M) or chemotherapy-related amenorrhea (A) at 2 to 5 years after diagnosis using serum AMH and chronological age at cancer diagnosis as predictor variables. The primary cutoff values are both for AMH, with below 3.8 pmol/L predicting amenorrhea and above 20.3 pmol/L predicting ongoing menses. Between these AMH levels there is an age threshold at 38.6 years, above which amenorrhea is predicted and below which ongoing menses are predicted. The classification schema has sensitivity 98·.2% (1 of 55 subjects known to have developed amenorrhea misclassified as having ongoing menses) and specificity 80.0% (4 of 20 subjects with known ongoing menses misclassified as amenorrhoeic). After 10-fold cross-validation this schema represents the optimal compromise between good fit to the data used to construct it, and low estimated error when used as a predictive model. Reprinted from (27) with permission.

MEASURING AMH AFTER CANCER TREATMENT

AMH Is a Measure of Current Ovarian Function in Post-Treatment Cancer Survivors

It is recognized that recovery of ovarian function after chemotherapy (as reflected in resumption of menses) varies by age and by diagnosis/treatment (33, 34). Younger women show a more rapid recovery, as do those treated for lymphoma compared to breast cancer, and recovery can take 2 years, or occasionally longer. AMH levels show this recovery. When modeling AMH in cancer survivors, levels are initially low immediately post-treatment then rise to peak between 2 and 3 years later (35). Prospective studies in women with breast cancer (median age 41), as discussed above, show both a marked fall versus pretreatment levels and minimal recovery of AMH levels over several years thereafter (25), while a comparable study in younger women with lymphoma (mean age 24) showed a clear divergence of the pattern of AMH levels by different chemotherapy regimens, with robust recovery of AMH levels in women treated with ABVD versus very limited recovery in women treated with high doses of alkylating agents (36).

With AMH levels drawn post-treatment as a reflection of current ovarian function in those patients, age, AMH at the end of treatment, and BMI are factors associated with the rate and extent of recovery in AMH following treatment. In addition to younger age, having a higher AMH at the end of treatment is associated with greater and faster recovery (32), and higher BMI may also be related to shorter time to recovery (31).

Following recovery after cancer treatment, AMH will again decline as it does in all women. Although this is a challenging aspect of the subject to study, whether the rate of decline is affected by prior chemotherapy has been investigated. In an

analysis of the rate of decline of AMH in 170 cancer survivors aged 15 to 39 years over a 2-year period, the rate of decline in AMH was similar to that in similarly aged controls, albeit at lower levels overall, and in both younger groups was much slower than in older women, aged 40 to 50 years (37). A second study reported the slope of change in AMH over approximately 3 years in long-term childhood cancer survivors (median of 16 years since cancer treatment) was similar to women without cancer (38). Taken together, although AMH levels will on average be lower in a cancer survivor than a woman without cancer, there may be a long duration of relative stability with a plateau over 10 to 15 years prior to decline rather than a rapid decline for many young women who are cancer survivors (35). This is very reassuring for such patients, although additional detail is needed to confirm that this applies across the range of AMH levels.

The Relationship Between Cancer Treatment and Ovarian Function is Reflected by AMH, and Is Modified by Age

In a recent analysis of recovery of ovarian function following treatment for Hodgkin lymphoma, it was confirmed that women treated with ABVD overall showed a complete recovery of AMH levels, in contrast to those treated with BEACOPP (**Figure 2**) (39). However, within the ABVD treated group recovery of AMH levels was markedly reduced to approximately 35% of pretreatment values in women aged over 35, whereas it was complete in younger women. This was not related to pretreatment AMH levels, thus young women with a low AMH at diagnosis showed a good recovery whereas older women with a higher AMH did not, thus the effects of age may be indicative of other aspects of ovarian aging, perhaps affecting the stroma or vasculature, and their damage by chemotherapy (40). The effect of age on recovery of ovarian function was also demonstrated in FSH levels, which were slower to return to normal in women aged over 35 than in younger women (39). This differential relationship between chemotherapy and post-treatment AMH levels by age in women with Hodgkin lymphoma is in contrast to data in women with breast cancer, where no effect modification has been observed. Potential explanations include the overall older age of the breast cancer population, with the majority in their late 30s and early 40s in most studies, or it may reflect the gonadotoxicity of chemotherapy with overall much less recovery of AMH in women treated with breast cancer chemotherapy regimens.

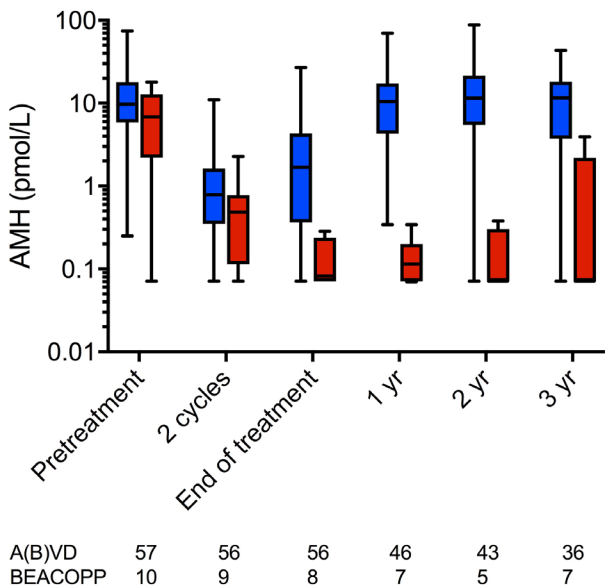

FIGURE 2 | AMH concentrations at pre-chemotherapy, after two cycles of ABVD, at end of treatment and at 1, 2, and 3 years after chemotherapy. Blue: women treated with ABVD throughout; red, women treated with BEACOPP after two cycles of ABVD. Boxes are median and IQR, whiskers indicate range. AMH is plotted on a log10 scale to show the low concentrations during and after BEACOPP more clearly. Numbers of subjects at each time point indicated below. Reprinted from (39) with permission.

Indeed, age modified the relationship between gonadotoxicity and post-treatment AMH trajectories in a large cancer survivor cohort (35). The trajectory for high gonadotoxicity (high dose alkylators, pelvic radiation, transplant) had a noticeably steeper decline after its initial rise in the first 2 to 3 years since cancer treatment, compared to the trajectories of the moderate and low gonadotoxicity groups which showed a prolonged plateau. In the same cohort, survivors who were older than age 30 at diagnosis exhibited consistently lower AMH trajectories compared to those younger than 25 years and those between ages 25 and 30 years. A test of interaction between age at treatment and gonadotoxicity was statistically significant, and data suggested that the protective effects of younger age on ovarian reserve when exposed to high gonadotoxicity treatments becomes diminished in the latter 20s.

Importantly, in the present context, recovery of AMH after chemotherapy does not reflect an increase in the ovarian reserve, but a recovery in the population of growing (AMH-producing) follicles in the functional ovarian reserve. Similarly AMH levels are reduced in healthy women taking hormonal contraception (41), and are also reduced in women with some cancer diagnoses, before treatment as discussed above.

Acute and Long-Term AMH Levels as Measures of Cancer Treatment Gonadotoxicity

Many cross-sectional studies clearly show that treatment type is related to AMH levels, both acutely and over the long-term. The first such study to show this was in young women who had been treated for childhood cancer, but who still had regular menstrual cycles thus overtly had normal ovarian function (42). These women were shown to have reduced AMH levels compared to age match controls and this key finding of the added value of AMH in assessment of post-treatment ovarian reserve has subsequently been replicated in a large number of studies across a range of diagnoses. Subsequent studies showed that AMH was markedly low following treatment for breast cancer (43, 44), following alkylating agent therapy and treatments associated with bone marrow transplantation (45), in women treated for Hodgkin lymphoma in childhood where a relationship with dose of alkylating agent was identified (46), and across a range of diagnoses in childhood and young adult

cancer survivors (47–50), confirming relationships with dose of alkylating agent and pelvic radiotherapy. The limitation of cross-sectional studies is that they shed little light on the pattern of change of ovarian function post treatment.

More limited prospective data are available. In a pediatric cohort of 22 girls with a range of diagnoses and aged 0.3 to 14 years, AMH fell progressively with each course of chemotherapy becoming undetectable in approximately half the group (51). The key finding was that initial fall of AMH levels and recovery varied by treatment regimen, with those treated with regimens assessed as having low/moderate gonadotoxicity showing recovery to pretreatment AMH levels, whereas those treated with high risk regimens (containing high doses of alkylating agents, or with pelvic radiotherapy) showed lower levels at the end of treatment and minimal or no recovery. Inhibin B and FSH were of no value in discriminating these treatment effects. This may allow improved assessment of girls at pre- and peri-pubertal ages, and support timely treatment for induction of puberty where there is clear and early evidence of absent ovarian function.

Variation in AMH levels by treatment also suggest that AMH may serve as a biomarker of gonadotoxicity. Acutely, between cancer diagnosis and end of treatment, AMH levels fell more in those exposed to alkylating chemotherapy exposure, compared to those not exposed to alkylators (32). Longitudinally, the rate of recovery in AMH did not vary by alkylator exposure, but this could be limited by sample size. Recently, using a hybrid cross-sectional and prospective cohort design, the post-treatment trajectory of AMH was modeled based on data from 763 patients with common cancers (**Figure 3**) (35). The magnitude of AMH recovery and duration of plateau was less for those whom underwent highly gonadotoxic therapies, compared to low or moderate gonadotoxic treatment groups. This study also used AMH measurements in dried blood spots collected at home: this technique may be of value to pursue large-scale clinically important questions.

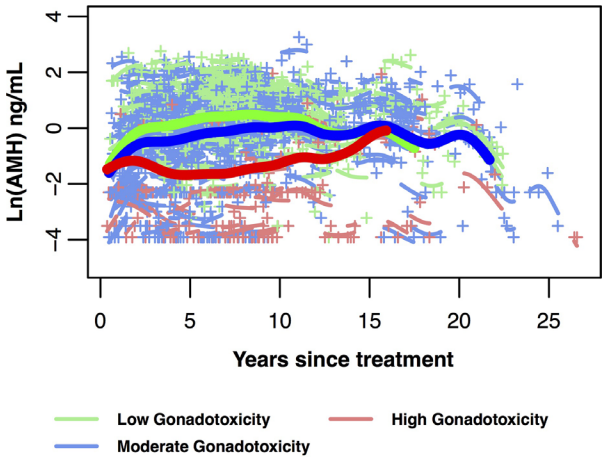

FIGURE 3 | AMH trajectories in 718 post-treatment AYA cancer survivors ages 25 to 40 years at AMH measurement. Data are divided into three gonadotoxicity groups, predicted mean log-transformed AMH trajectories over years since cancer treatment (bold lines with green for low, blue for moderate, red for high gonadotoxicity). Mean curves are truncated when the number of individual participants remaining in the group is fewer than 10. Individual log-transformed AMH levels (+) and predicted trajectories (short lines related to +) also are depicted in the same colour as their gonadotoxicity group. This figure is original and based on data from (35).

Post-Treatment AMH and Diagnosing Ovarian Insufficiency

The accurate diagnosis of permanent ovarian insufficiency is of considerable value in patients treated for cancer beyond fertility considerations. Ovarian function is also of importance in hormone dependent breast cancer as it may impact on choice of endocrine therapy. The use of aromatase inhibitors is now established to improve survival in post-menopausal women with breast cancer (52), but these drugs require concurrent ovarian suppression with GnRH agonists in pre-menopausal women. Uncertainty in identifying which women have become truly post-menopausal following treatment when they were pre-menopausal beforehand results in reluctance to stop GnRH agonist ovarian suppression even when in reality in an individual patient it may not be required. In the context of the normal menopause, initial studies showed that AMH became undetectable several years before the menopause, and therefore the assays available at that time were clearly insufficiently sensitive to have confidence in the use of AMH as an accurate diagnosis of POI following chemotherapy. However, currently available assays, both automated (Roche and Beckman Coulter) and the manual PICO assay (Ansh laboratories), have markedly improved sensitivity, and data on the relationship between AMH and natural menopause are becoming clearer (53), although age remains an important determinant of the accuracy of prediction. In a re-analysis of 98 blood samples taken 2 years after breast cancer treatment using the PICO assay, undetectable AMH was an extremely good predictor that ovarian function would not recover over the following few years, with 96% specificity (54). Subsequently the value of an undetectable AMH level, using the Roche automated assay, was shown to be an accurate diagnostic test for POI at 2 years following diagnosis with 100% sensitivity and 73% specificity (55), thus it appears that after allowing 2 years for any potential recovery of ovarian function, an undetectable AMH level is indeed an accurate index that recovery of ovarian function is very unlikely. While these data are exciting, it is important to note that substantial inter-assay differences remain with regard to AMH assays (56), which may be of particular importance at the lower limit of detection, and thus, the generalizability of cut points is assay-dependent. It is also the case that a proportion of women, perhaps as high as 10% in a young population (57), may have episodes of vaginal bleeding after more than 2 years of post-cancer amenorrhea, possibly reflecting transient ovarian activity.

AMH levels have been shown to be not influenced by tamoxifen co-administration, but they are suppressed by GnRH agonist administration over a period of several months (25), thus that needs to be taken into account in analyzing AMH levels in that context. Given the high predictive value of an undetectable AMH level at 2 years, the

question is therefore how early after chemotherapy can AMH be used to accurately identify permanent POI. In that same analysis of women with breast cancer, AMH analysis at the end of chemotherapy was also analyzed (55). Overall, in a group of 68 women, an undetectable AMH at end of chemotherapy had a sensitivity of 78% and specificity of 82% for prediction of POI at 2 years, giving a diagnostic odds ratio of 10.9. This surpassed the value of FSH, which while it had a very high sensitivity (inevitable given that it is part of the diagnosis of POI), the specificity was low, and the diagnostic odds ratio was only 5.5. However, in a sub-group analysis of women aged over 40, the accuracy of AMH at end of treatment was improved with a sensitivity of 91% and specificity 82%, giving a high diagnostic odds ratio of 42.8. It may therefore be that using these high sensitivity assays that an AMH assessment on completion of chemotherapy can accurately identify those women who will not show any recovery of ovarian function following chemotherapy, and this may be of value in determining the most appropriate adjuvant endocrine treatment, but this may be limited to women in their forties. More conservatively, breast cancer survivors who are amenorrhoeic without GnRH agonist suppression for 2 years after chemotherapy and have an undetectable AMH level may be candidates for aromatase inhibitor endocrine therapy without concurrent GnRH agonist, but subsequent vaginal bleeding would require reassessment of ovarian function. This requires prospective evaluation.

CONCLUSION

The above discussion clearly shows the value of pre- and post-treatment AMH in female cancer survivors for predicting post-treatment ovarian function, serving as a biomarker of treatment gonadotoxicity, and in the diagnosis of ovarian insufficiency, particularly when a sufficient time for early recovery of ovarian function has been allowed to elapse. Pretreatment analysis of AMH level is of value in predicting long term ovarian function in women with breast cancer but its value in other diagnoses and with other types of chemotherapy regimen, particularly when less gonadotoxic are unclear. The interaction with age in this respect is intriguing, and it is likely to require a greater understanding of these adverse effects of chemotherapy on the different compartments and cell types of the ovary. There are promising data that in certain sub-groups AMH may be of value shortly after completion of chemotherapy and ultimately this may be of value in guiding adjuvant endocrine therapy in some women with breast cancer. Current data suggest that AMH is not of value in predicting short term fertility in women following cancer treatment as shown both by specific analysis (58) and in individual cases within larger analyses (39), as is the case for women in the general population (59, 60). Unfortunately there remains a dearth of data regarding the use of AMH to estimate time to menopause in the post-cancer treatment setting and beyond that into whether AMH can help stage the process of reproductive senescence similar to in the general population (61). Data are lacking on AMH's relationship to non-reproductive late effects relating to estrogen deficiency such as bone health and potentially cardiovascular and cerebral vascular function. Larger prospective studies with these diverse end points are needed to clarify the key areas where AMH is of value in this context.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Role of AMH in Prediction of Menopause

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Anti-Müllerian Hormone (AMH) is produced by small antral follicles and has evolved over the past three decades as an assumed potential marker of the number of follicles in the human ovaries, also known as ovarian reserve. This quantitative measure, given the gradual decline over time and its non-replenishable feature, could be the dreamed marker for predicting the final exhaustion of ovarian storage: the post-menopause. This introductory chapter summarizes current knowledge with regard to the contribution of serum AMH measurements to predict age of normal menopause and critically discuss its potential in this regard. Furthermore, its predictive role in the context of menopause in association with several frequently occurring fertility disorders such as premature menopause, polycystic ovarian syndrome and endometriosis are discussed. Overall, while ovarian reserve markers including AMH are unmistakably related to age at menopause, they are insufficiently precise to inform on an individual's journey of ovarian aging.

Keywords: AMH, menopause, prediction, ovarian aging, reproduction

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INTRODUCTION

"Trying to predict the future is a loser's game" (Ken Lui). Yet in medicine, prediction of future events is commonly used as a means of stratifying people into high or low risk and as an aid for treatment individualization. For every woman, the occurrence of menopause is a given, but the age at which she will enter menopause varies widely and is normally distributed between the ages of 40 to 60 (1). The onset of the menopausal transition indicates that the pool of resting and developing follicles in the ovaries, also known as ovarian reserve, is nearly depleted. Measuring the remaining ovarian reserve prior to this event could theoretically thus provide a risk estimation for the timing of the onset of menopause.

True ovarian reserve, i.e. the number of resting primordial follicles, can only be measured through histological tissue analysis. Available ovarian reserve tests therefore serve as a proxy for true ovarian reserve. The antral follicle count (AFC) measures the number of developing antral follicles by ultrasound, which is correlated to the size of the resting follicle pool (2). Levels of follicle-stimulating hormone (FSH) start to increase secondary to the age-related decline of estrogen-producing developing follicles. Rising FSH levels thus indicate a late stage of ovarian aging. Anti-Müllerian hormone (AMH) is produced by small developing, mostly antral, follicles. As these follicles are not yet responsive to FSH, AMH levels remain relatively stable throughout the

menstrual cycle. In comparative studies, AMH was found to be the most favorable ovarian reserve marker for the prediction of age at menopause (3, 4).

AGE AT MENOPAUSE PREDICTION

The majority of research efforts spent on the prediction of menopause stems from a desire to predict the duration of the reproductive lifespan. The rationale is usually that this enables a woman to gain information on the remaining time period she may have to become pregnant. Another objective could be to base a treatment strategy, such as ovarian surgery, on predicting the remaining years until the onset of menopause in a perimenopausal woman experiencing debilitating symptoms such as heavy menstrual bleeding or hot flashes. Whichever the objective, studies on the topic of menopause prediction require the assessment of desired predictors (such as age and AMH) at a baseline time point and a longitudinal follow-up period during which the event of interest (menopause) is recorded. Naturally, the study question dictates how much follow-up time is required. Statistical analysis of menopause prediction occurs through the development of prognostic models and a time-to-event analysis with a binary outcome (menopause or no menopause during follow-up), or the prediction of menopausal age as a continuous outcome.

Both statistical approaches have previously been utilized in menopause prediction studies. In all studies to date, AMH has proven to be a significant predictor for time to menopause or age at menopause (3, 5–12). The effect measure of AMH was presented in various ways; as an example, one unit decline in $_{log}$AMH was associated with a 1.75-year earlier menopause (13), every unit increase in AMH was associated with decrease in the chance of becoming postmenopausal during follow-up illustrated by a hazard ratio (HR) of 0.092 [95% CI 0.025-0.340] (7); women in the lowest AMH quartile had a 8.39 times higher risk of becoming postmenopausal during follow-up compared to women in the highest quartile (14). Prediction of individual age at menopause with the inclusion of AMH in the prediction model furthermore led to a similar distribution of predicted and observed ages at menopause (6, 9) and the observation that women in low age-specific AMH percentiles generally reached menopause at an earlier age than women with high age-specific AMH percentiles (7, 10).

METHODOLOGICAL PITFALLS OF MENOPAUSE PREDICTION

As can be expected, these results created much anticipation of the promise of AMH as a determinant of reproductive age. Indeed, a literature review of variables of menopause prediction concluded that AMH was the most promising predictor available (4). However, as the authors mention, this conclusion can be somewhat nuanced by a further exploration of the statistical analyses of these studies. Aside from statistical significance of an included predictor variable, the performance of prediction models should also be considered in their interpretation. In a pair of two women, the C-statistic provides a measure of how often the prediction model correctly identifies who will become postmenopausal during a set time period, based on their AMH levels (and other additional predictors in the model). Overall, the C-statistics in the aforementioned prediction studies were high (all above 80%), thus expressing a high degree of model discrimination. The addition of AMH to a similar model with age led to an overall improvement of the C-statistic, specifically from 84% to 92% (6); 87% to 90% (7), 85% to 92% (3) and 84% to 86% (10). While this improvement should be acknowledged, it is also clear that the added predictive effect of AMH to an already well-performing model is in fact modest. The C-statistic was not reported in two studies (5, 8).

Another factor to consider is the non-proportional predictive effect of AMH with age. With increasing age, the predictive capacity of AMH on top of age alone decreases (10). This may be interpreted in the sense that a regularly cycling woman at age 43, based on these characteristics alone, already has a far lower *a priori* chance of early menopause in comparison to a woman at age 30. Although this seems like an encouraging finding in menopause prediction for younger women, the downside is that the predictive *accuracy* of AMH is lower for women at younger ages. Thus, while AMH contributes more to a prediction model for a younger woman seeking to know whether she may enter menopause at a relatively early age, the predicted age range in which she will enter menopause is wider. A further methodological issue at play here is the relative lack of inclusion of women below the age of 30 years in the cohorts utilized in the aforementioned studies. The wide prediction intervals may therefore, at least in part, be influenced by insufficient statistical power.

One potential way to work around the limitation of menopause prediction with a single AMH measurement is to get an indication of the speed of the AMH decline. This may thus provide information on the kind of ovarian reserve decline trajectory a woman is on, with a swift decline theoretically leading to an earlier age at menopause. In a longitudinal analysis of 5 AMH measurements spanning a time period of 20 years, the speed of AMH decline was associated with AMH levels and found to vary with age (15). This suggests that a one-size-fits-all approach to AMH decline may be flawed. After the age of 25, knowledge of the prior 5-year AMH decline rate did not lead to improved C-statistics in comparison to the AMH level alone in the same cohort (11). In concordance with prior findings, AMH measurement at 20 and 25 years was associated with poorer C-statistics in comparison to AMH measurement at 30 years (62, 64 and 70% respectively). A more recent cohort study did find an improvement of C-statistics in menopause prediction with the addition of the AMH decline rate, calculated over a span of approximately 18 years: 70% to 78% (12). The latter finding has limited clinical applicability however, as this cannot be extrapolated to a short-term decline rate due to the large variation in AMH decline rate over time. It is unlikely that a

woman seeking information on her future menopausal age, especially with the objective of family planning, will have the patience to wait 18 years for an improved prediction model.

Lastly, there are currently several AMH assays available for clinical use. These include the Gen II (Beckman Coulter), picoAMH (AnshLabs), AMH ELISA (AnshLabs), Elecsys (Roche) and Access (Beckman Coulter). Each assay has a different range of detection and sensitivity, which impedes the direct comparison and formulation of cut-off values of absolute serum AMH levels measured by different assays (16). Naturally, this means that if a well-performing model would be developed for AMH, its results would only directly apply to AMH measurements performed with the same assay, or require the application of a correction factor with an added risk of inaccuracy.

MENOPAUSE PREDICTION IN CLINICAL SUBGROUPS

Primary Ovarian Insufficiency

Primary ovarian insufficiency (POI) is defined by the permanent cessation of menses before the age of 40 years with substantially elevated levels of follicle-stimulating hormone (FSH). The situation when there is still a menstrual cycle but already elevated levels of FSH in the early follicular phase is called imminent ovarian insufficiency with the elevated FSH as the result of limited ovarian inhibin B feedback (17). Elevated levels of FSH are an irrefutable hormonal hallmark of reproductive aging. Unfortunately, longitudinal studies have shown that a markedly elevated FSH is a relatively late predictor of the menopausal transition, since increasing values only occur about 10 years before the menopause, which is probably also when infertility begins to prevail (18). Longitudinal studies have shown that inhibin B correlates with age only during a relatively short period before the menopause transition (19). A decrease in inhibin B seems the most important and earliest factor that plays a role in the elevation of early follicular phase FSH. Low or unmeasurable inhibin B levels theoretically could be used to indicate that the menopause is imminent. Unfortunately, its role as an early predictor is also limited. Thus, both elevated FSH and a declined Inhibin B seem appropriate predictors, but only just prior to the occurrence of menopause.

Current data indicate that measurement of AMH is a more accurate indicator of POI in many situations with diagnostic validity, and perhaps may facilitate more timely diagnosis although there are scarce data regarding prediction of POI far in advance. Within the prospective Nurses' Health Study II cohort each 0.10 ng/ml decrease in AMH was associated with a 14% higher risk of early menopause supporting the potential utility of AMH as a clinical marker of early menopause in otherwise healthy women (20). However, due to inadequate precision, the ability of AMH to accurately predict the distant onset of POI seems as unreliable as for any age at menopause (21).

Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is a highly prevalent reproductive endocrine disorder characterized by a varying degree of hyperandrogenism, polycystic ovaries and anovulation which has some remarkable features with regard to reproductive ageing. Typically, women with previous anovulation almost all become ovulatory by the age of 40 (22). There are indications that PCOS is associated with a substantial delay of menopause by more than 4 years compared to regularly ovulating women. Prediction of age at (23) menopause using AMH in women with PCOS would correspond to an average extension of the reproductive lifespan by two years (24). With the assumption that higher AMH levels relate to later menopause, it is not unreasonable to interpret the substantially higher serum AMH levels in women with PCOS as a prelude to a later age at menopause. This then supports the notion that these higher AMH levels relate to a preexisting larger pool of follicles that exhausts later in life, aside from biochemical mechanisms that promote AMH secretion. Currently, substantially sized long-term follow-up studies relating previously measured AMH at a younger age to the actual age at menopause some years later in women with PCOS are not available.

Endometriosis

Another condition of the reproductive system with concerns regarding fertility and age at menopause is endometriosis, and in particular ovarian endometriosis. Intuitively, an ovarian endometrioma may potentially affect ovarian reserve through its intrusion of ovarian tissue. To date only a few studies (25, 26) addressed this issue. According to Streuli et al. (25), endometriosis and ovarian endometriomas are not singularly related to lower AMH levels. In contrast, Uncu et al. (26) found that compared to controls, women with endometriomas did have lower AMH levels prior to surgery. Thus, there is still controversy here. A recent systematic review which compared AMH levels between women with uni- and bilateral endometrioma found no difference, which challenges the concept of damage to ovarian reserve by endometriomas. Surgical intervention is more consistently related to a sustained decline of ovarian reserve markers (27). Whether these lower AMH levels after the surgery predict earlier menopause, as may be expected, remains to be established. There are indications that this may be the case (28).

Iatrogenic Ovarian Reserve Impairment

Women who have undergone treatment that may affect the pool of dormant primordial follicles represent a different category with regards to ovarian reserve measurement. In childhood cancer survivors, AMH levels appear to adequately reflect the ovarian reserve potential after gonadotoxic chemotherapy (23). Women who exhibited signs of preserved ovarian reserve after finalizing chemotherapy retained proportionally similar ovarian reserve status after 10 years of follow-up, which suggests that the decline of ovarian reserve may not be significantly altered (29). Indeed, in a longitudinal population study of childhood cancer survivors with detectable post-treatment AMH levels, the decline rate of AMH was very much comparable to that of a control population (30). It remains to be determined whether the prediction of age at menopause, albeit with a range spanning several years, may ultimately be feasible in this group of women

facing reproductive decisions at a relatively early age. Of note, AMH levels are reduced in girls with newly diagnosed cancer even before the cancer treatment has started and it is suggested that possibly in relation to this impaired DNA repair mechanisms are involved that also seem in part to be involved in determination of age at menopause (31–33).

IMPLICATIONS OF MENOPAUSE PREDICTION

There is ample discussion as to whether and for whom menopause prediction can be applied in clinical practice. As previously highlighted, there are several groups of women who may benefit from a personalized estimate of the duration of their reproductive lifespan. For example, a young woman, considering whether she should opt for family or career first, could base her decision on potential biological restrictions. Indeed, younger women with a future desire for family planning were reportedly interested in testing for premature menopause (34). This willingness is capitalized on by companies offering 'fertility' tests, which often include an estimate of ovarian reserve. As discussed, while ovarian reserve markers are unmistakably related to age at menopause, they are insufficiently indicative of an individual's journey of ovarian aging. The use of ovarian reserve markers for long-term predictions could therefore either lead to a false sense of security or unnecessary alarm. Similarly, a woman with a familial risk of POI may not benefit from an AMH measurement in the long-term, although the finding of a nearly depleted follicle pool may prompt her to take action in the short term. Still, the knowledge level of young women on their current and future fertility and the effects of increasing age is poor. Fertility tests may draw attention to these themes with the effect that women get informed on these topics and will be aware of the potential risks. Also, there is a need to understand and probe the way young women would handle results of fertility tests: if the early menopause (before age 45) hazard is 25% instead of 5%, based on the AMH test: what will young women do with such a result? This needs to come with information on the other player in this important field: average oocyte quality, which currently can only be captured by female age, but at the same time may highly vary within age categories. Understanding the interplay between follicle number and oocyte quality will help to understand that, in order to have a chance of at least 90% to naturally realize a two-child family, couples should start trying to conceive when the female partner is 27 years of age or younger (35). The role of AMH as a marker of ovarian aging in women with PCOS and endometriosis requires further elucidation, as there are multiple processes at play that may influence or be influenced by ovarian reserve in these heterogeneous disease entities.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Translational Physiology of Anti-Müllerian Hormone: Clinical Applications in Female Fertility Preservation and Cancer Treatment

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Background: Whilst the ability of AMH to induce the regression of the Müllerian ducts in the male fetus is well appreciated, AMH has additional biological actions in relation to steroid biosynthesis and ovarian follicle dynamics. An understanding of the physiology of AMH illuminates the potential therapeutic utility of AMH to protect the ovarian reserve during chemotherapy and in the treatment of female malignancies. The translation of the biological actions of AMH into clinical applications is an emerging focus of research, with promising preliminary results.

Objective and Rationale: Studies indicate AMH restrains primordial follicle development, thus administration of AMH during chemotherapy may protect the ovarian reserve by preventing the mass activation of primordial follicles. As AMH induces regression of tissues expressing the AMH receptor (AMHRII), administration of AMH may inhibit growth of malignancies expressing AMHR II. This review evaluates the biological actions of AMH in females and appraises human clinical applications.

Search Methods: A comprehensive search of the Medline and EMBASE databases seeking articles related to the physiological functions and therapeutic applications of AMH was conducted in July 2021. The search was limited to studies published in English.

Outcomes: AMH regulates primordial follicle recruitment and moderates sex steroid production through the inhibition of transcription of enzymes in the steroid biosynthetic pathway, primarily aromatase and 17α-hydroxylase/17,20-lyase. Preliminary data indicates that administration of AMH to mice during chemotherapy conveys a degree of protection to the ovarian reserve. Administration of AMH at the time of ovarian tissue grafting has the potential to restrain uncontrolled primordial follicle growth during revascularization. Numerous studies demonstrate AMH induced regression of AMHR II expressing malignancies. As this action occurs *via* a different mechanism to traditional chemotherapeutic agents, AMH has the capacity to inhibit proliferation of chemo-resistant ovarian cancer cells and cancer stem cells.

Wider Implications: To date, AMH has not been administered to humans. Data identified in this review suggests administration of AMH would be safe and well tolerated.

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Administration of AMH during chemotherapy may provide a synchronistic benefit to women with an AMHR II expressing malignancy, protecting the ovarian reserve whilst the cancer is treated by dual mechanisms.

Keywords: anti-Müllerian hormone, fertility preservation, steroid biosynthesis, aromatase, ovarian cancer, chemotherapy

INTRODUCTION

In the late 1940s, a series of studies was conducted by Jost (1, 2) in which testosterone implants were surgically introduced into female rabbit fetuses. Whilst these experiments indicated that testosterone was capable of inducing the development of male reproductive structures including the penis and scrotum, testosterone was not shown to cause the regression of Müllerian structures. On the other hand, grafting of testicular tissue in close proximity to the ovary in female rabbit fetuses resulted in the regression of the Müllerian ducts on the ipsilateral side to the testicular tissue. This led to a proposition by Jost as to the existence of a Müllerian inhibiting substance produced by the testis (3). This substance was later identified as a testicular glycoprotein, anti-Müllerian hormone (AMH, Müllerian inhibiting substance), for which both the bovine and human genes have been isolated (1).

The AMH gene is located on chromosome 19p13.3 (4). It has five exons and four introns (1). Transcription and cleavage of this gene results in a 535 amino acid AMH protein, comprised of a 426 amino acid N-terminal prodomain and a 109 amino acid C-terminal domain that conveys the biological activity of the molecule. AMH proteins bind together *via* disulfide bonds to form homodimers, but do not become active until cleavage of the prodomain occurs (5–7). A cleavage motif exists at the arginine-serine site at residues 427-428 (5, 8–10). Mutations that block cleavage of the AMH protein at this site destroy its biological activity (10).

The prodomain is essential for correct protein folding and facilitates the dimerization of the carboxy-terminal growth factor domains. Even after cleavage of the prodomain, there is frequently a persistent non-covalent binding between the prodomain and the active protein. AMH is a member of the transforming growth factor β (TGF-β) family. This family is comprised of thirty-three members that include activins, inhibins, bone morphogenetic proteins (BMPs) and growth differentiation factors (11). In other TGF-β family members, the association of the prodomain with the active growth factor domain may inhibit binding of the active hormone to its receptor as the prodomain can form a shield over the growth factor domain (12). However the non-covalent association between the prodomain and carboxy-terminal growth factor domain of AMH that persists after cleavage greatly potentiates its activity (13), with the prodomain disassociating with the carboxy-terminal domain only after its engagement with the receptor (14).

In line with other members of the TGF-β family, AMH acts *via* a heteromeric serine/threonine receptor complex. The gene for the AMH receptor II (AMHR II) is located on chromosome 12q13 (15). The transcription of this gene results in a protein consisting of 573 amino acids. The AMHR II conveys the biological specificity of the receptor complex. It has an extracellular domain for ligand binding, a transmembrane domain and an intracellular domain with the capacity for serine/threonine kinase activity (6). A unique AMH receptor I (AMHR I) has not been identified. It appears that the function of AMHR I is conducted by a receptor that is shared with bone morphogenetic proteins (BMPs). The ALK2, ALK3 (BMPR-1A) and ALK6 (BMPR-1B) receptors have been proposed as AMHR I receptors (11, 16–19).

METHODS

The EMBASE and MEDLINE databases have been searched using search the search terms "Anti-Müllerian hormone" OR "Müllerian inhibiting substance" OR "Müllerian inhibiting hormone" OR "Müllerian inhibiting factor" in conjunction with the following terms: "physiology", "steroid synthesis", "fertility preservation" and "neoplasms" in combination with additional keywords associated with specific topic areas. Searches were limited to articles written in English but were not limited by date. Additional references were obtained through an analysis of the references in key articles.

THE INCREASING IMPORTANCE OF FERTILITY PRESERVATION

Globally over 8.5 million women were diagnosed with cancer in 2018. This number is projected to increase by 56.8% by 2040 to over 13.5 million women (20). Whilst the primary drivers of this increase are the growing size of the world's population, extended life expectancy and the ageing population, even after standardization for age incidence rates have slowly increased in the last two decades. In 2017 the worldwide cancer incidence rate was 306.75 per 100,000 having risen from 296.09 per 100,000 in 1990. This is largely due to the rapid progress in preventing mortality from diseases that previously killed young people, such as infectious diseases (21).

There is a clear correlation between age and cancer incidence, with almost 80% of the new cases of cancer being diagnosed occurring in women 50 years of age or older. Despite this, a large number of pre-pubertal girls and women of reproductive age are diagnosed with cancer. An estimated 1,358,073 females aged 0-44 years were diagnosed with cancer in 2018, the most

commonly diagnosed type being breast cancer, with 417,091 cases of this cancer being reported (20).

Within the 0-44 year age group, the strong correlation between cancer incidence and age is evident. Data from 2017 demonstrates an exponential rise in the number of women diagnosed with cancer in each decade of life, increasing from 272,971 cases in women aged 20-29 years, to 683,938 cases in women aged 30-39 years, and to 1,271,485 cases in women aged 40-49 years. This has important ramifications regarding the potential demand for fertility preservation as childbearing is increasingly being deferred to an older maternal age. In Australia the proportion of women having babies at less than 30 years of age halved from 80% in 1975 to 40% in 2018, with a concurrent quadrupling of the number of women aged 35 years or older giving birth, increasing from 6% in 1975 to 24% in 2018 (22). The experience of other Western countries is comparable – in England and Wales, the mean maternal age rose from 26.4 years in 1975 to 30.6 years in 2018 (23); in the United States of America, the mean maternal age at first birth rose from 21.4 years in 1970 to 26.3 in 2014 (24, 25); in the European Union, the mean maternal age rose from 29.0 years in 2001 to 30.8 years in 2018 (26).

Concurrent with these changes, cancer death rates have fallen. The worldwide age-standardized cancer death rate improved by 15% in the period 1990-2017, with a several countries including the USA, Canada, UK, Germany, France, Italy, Switzerland, Japan, Singapore and Australia reporting falls in their age-standardized cancer death rates of over 20% (27). Five-year survival rates for most cancers have also markedly improved in recent decades; in the USA for example, the overall five year survival rate rose from 50.3% in 1970-77 to 67.0% in 2007-2013.

With improving survival rates, there is an increasing focus on quality of life, of which fertility is of utmost importance. Qualitative studies consistently reiterate the importance of fertility for pre-menopausal women diagnosed with cancer (28–31). The finding that women were willing to alter their cancer treatment in order to preserve their fertility has repeatedly been reported. In a European study of 389 women aged 35 years or less at the time of breast cancer diagnosis, 8.2% of women stated that they would refuse chemotherapy if it would reduce their chance of being able to have children in the future (30); an international study of 657 women aged 40 years or less at the time of breast cancer diagnosis reported 29% of women stated that concerns regarding fertility influenced their cancer management decisions (28); an American study of 620 women aged 40 years or less at the time of breast cancer diagnosis reported that 51% of women were concerned about becoming infertile as a result of their cancer treatment, with concerns regarding fertility affecting their cancer treatment decisions in 26% of women. In this latter group of women, four women (1%) refused chemotherapy completely and 12 women (2%) altered the chemotherapy regime they would accept due to concerns regarding infertility (29).

The need for specialist involvement does not end with the instigation of cancer treatment. Fertility issues can become increasingly important to women after the initial shock of their cancer diagnosis dissipates (31). The rising incidence of cancer in pre-menopausal women and improvements in long-term survival rates, act together to increase cancer prevalence. It is estimated that the global prevalence of women aged 15-49 years with history a cancer diagnosis has increased from 14.02 million in 1990 to 24.62 million in 2017 (27). Consequently, fertility preservation strategies prior to the commencement of cancer treatment, and the ongoing management of women with a history of cancer, has transpired as a crucial component of care provided by reproductive endocrinologists. Demand for specialist care is likely to evolve in conjunction with continued advances in early cancer detection and improvements in cancer survival rates.

THE ACTION OF AMH IN THE STEROID BIOSYNTHETIC PATHWAY

AMH has several actions in the steroid biosynthetic pathway, as indicated in **Figure 1**. Whilst AMH does not suppress basal enzymatic expression, it has the capacity to mitigate hormonally mediated elevations in transcription thus acting as a regulator of sex steroid production.

The most extensively documented action of AMH in the steroid biosynthesis pathway relates to its inhibition of transcription of CYP19A1. CYP19A1 (aromatase) belongs to the cytochrome P450 superfamily and is responsible for the aromatization of androgens (androstenedione and testosterone) to oestrogens (oestrone and oestradiol respectively). *In vitro* studies of murine, porcine and human granulosa cells have demonstrated that addition of AMH to culture media does not alter the basal expression of aromatase (32–36) but does inhibit increases in aromatase mRNA transcription mediated by FSH (32–34, 36–39), LH (38) and a combination of FSH and LH (36).

FSHR and LHR are G-protein coupled receptors. The binding of FSH and LH to their respective receptors results in signal transduction modulation, at least partially through the activation of adenylyl cyclase and production of 3'5'-cycleic adenosine monophosphate (cAMP) as a second messenger (40). Thus the addition of cAMP or forskolin (a direct activator of adenylyl cyclase capable of inducing a rise in intracellular cAMP levels) mimics the action of gonadotropin downstream of FSHR and LHR. AMH has been shown to inhibit cAMP- and forskolin-mediated increase in aromatase expression (32, 35, 37–39).

As inhibition of aromatase prevents the conversion of androgens to oestrogens, inhibition of aromatase would logically be expected to decrease oestrogen and increase androgen concentrations. This has been demonstrated in one *in vitro* study in which the ovaries of sheep fetuses, obtained 29 days post-coitum, were cultured in the presence or absence of AMH, with androstenedione being used as a substrate for aromatization. Ovaries cultured in control media secreted oestradiol with only low concentrations of testosterone detected. Ovaries cultured in the presence of AMH showed a reversal of this pattern, with negligible oestradiol secretion and high testosterone concentrations detected, the latter approximating

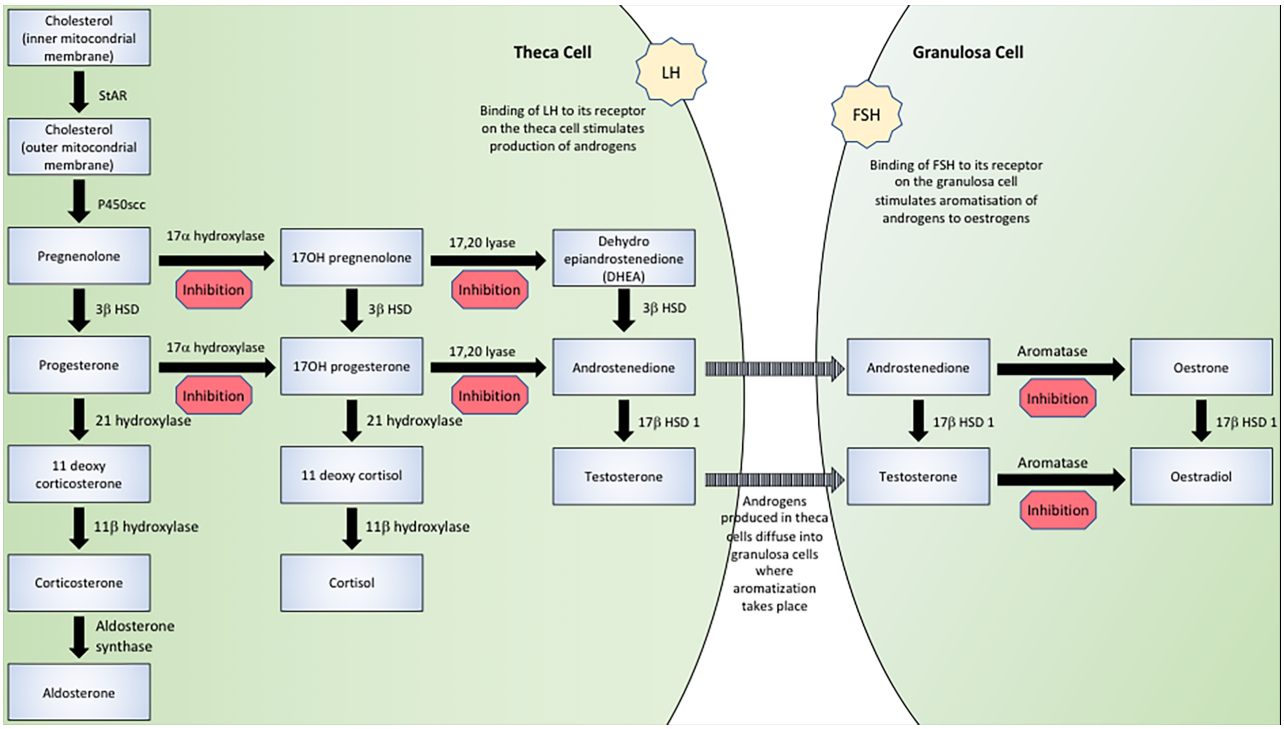

FIGURE 1 | The effect of AMH in the steroid biosynthetic pathway.

those detected in testicular culture controls. Assays of aromatase activity confirmed that the elevated testosterone and low oestradiol concentrations were secondary to reduced aromatase activity. These results were replicated by the same authors using 16-28 day post-coitum rabbit fetal ovaries (41).

Numerous *in vitro* studies report inhibition of FSH mediated increases in oestradiol by AMH with unchanged basal production of oestradiol (32, 34, 35, 38, 42). Supporting these findings is an *in vivo* study in which mice administered with 120ng or 300ng AMH for four weeks demonstrated decreased aromatase activity and decreased serum oestradiol concentrations (37).

However, the increase in testosterone production that would be expected with aromatase inhibition has not been consistently reported; conversely the majority of *in vitro* studies report *decreased* testosterone production in the presence of AMH (39, 43–46), a finding that has been replicated *in vivo* (44, 46, 47).

A study in which supra-physiological AMH concentrations in mice were generated through the administration of an adeno-associated virus serotype 9 (AAV9) vector reported decreased serum concentrations of both oestradiol and testosterone (48). Two studies of transgenic male and female mice engineered to overexpress AMH under the control of a metallothionein promoter reported significantly reduced serum testosterone concentrations in adult male transgenic mice compared to controls (47, 49); with one of these studies reporting an undetectable serum testosterone concentrations in both transgenic female mice and controls despite ovarian aromatase activity being significantly reduced in the transgenic mice, indicating testosterone does not accrue in AMH overexpressing female mice (47). A further study of male mice administered AMH *via* intra-testicular injection demonstrated decreased testicular interstitial fluid testosterone concentrations; Leydig cells obtained four hours post-injection were cultured for three hours with decreased testosterone production demonstrated in mice administered AMH (44). A final *in vivo* study in which rats were administered rAMH, reported a three-fold reduction in serum testosterone concentrations 24hrs after administration of AMH (46).

The repeated finding of decreased serum testosterone concentrations alludes to an additional inhibitory action of AMH at an earlier stage of the steroid biosynthesis pathway. Granulosa cells perform only the final stage (aromatization) of oestrogen production, the initial stages involving the conversion of cholesterol to androgens being performed in theca cells, as illustrated in **Figure 1**. Therefore *in vitro* studies of granulosa cells in which androstenedione is added to the culture media do not demonstrate a reduction in testosterone synthesis (41) as the addition of androstenedione effectively bypasses the early stages of steroid biosynthesis. *In vitro* studies in which androstenedione is not provided as a substrate (39, 43–46) and *in vivo* studies (44, 46–49), show decreased testosterone production.

Consistent with the supposition that AMH must inhibit sex steroid production at early stages of the biosynthetic pathway are findings of decreased mRNA expression of CYP17, the gene encoding 17α-hydroxylase/17,20-lyase, which is responsible for the conversion of pregnenolone/progesterone to 17OH-pregnenolone/17OH-progesterone to DHEA/androstenedione

respectively (35, 43–46, 49). In an *in vitro* study, researchers cultured MA-10 cells from a Leydig cell tumour line in the presence of AMH and reported a striking ten-fold reduction in testosterone secretion after two days of culture, with a modest 40% reduction in progesterone secretion noted. Expression of CYP17 dropped to undetectable levels in the presence of AMH (45). An *in vivo* study in which adult male mice were injected with either hCG alone or hCG and AMH reported a 9-fold reduction in serum testosterone concentrations 24 hours after injection with AMH, with a slight (statistically significant) decrease in 17OH-progesterone, suggesting strong inhibition of 17,20-lyase with lesser inhibition of 17α-hydroxylase activity. Serum progesterone concentrations were unchanged (46).

Steroidogenic acute regulatory protein (StAR) delivers cholesterol from the outer to the inner mitochondrial membrane, which is the rate-limiting step in steroid biosynthesis (50). Several studies have investigated the effect of AMH on expression of StAR, with conflicting results. There are similar inconsistencies between studies that explore the impact of AMH on P450scc and 3βHSD. Some studies have reported decreased P450scc mRNA expression secondary to AMH (36, 45, 46, 49), whilst other studies report unchanged expression (36, 46). A species specific effect has been postulated after a study demonstrated a reduction in P450scc mRNA expression in rats but not in mice (46).

A final means by which AMH may influence the production of steroid hormones is by its capacity to alter hormone receptor expression. Several studies have reported either reduced expression of LH receptor (LHR) mRNA (37, 44, 49) or a blunting of an LH mediated increase in LHR expression (35). Most studies report unchanged expression of FSH receptor (FSHR) mRNA (32, 33, 37, 38) and the progesterone receptor (PR) mRNA in the presence of AMH (37). One study in which AMH was administered to mice reported unchanged androgen receptor (AR) mRNA in pre-pubertal mice and decreased AR mRNA expression in pubertal mice (37).

Observational human studies support a role of AMH in human sex steroid production with an inverse relationship between serum AMH and testosterone concentrations documented in males (51, 52) Serum AMH concentrations in males are elevated at birth and remain high until puberty when a precipitous drop occurs (51–54); serum testosterone concentrations mirror this pattern (51, 52). As AMH diminishes testosterone production due to its inhibitory action on P450 17α-hydroxylase/C_{17-20} lyase (39, 43, 45), the precipitous drop in serum AMH concentration occurring at puberty would release the inhibitory constraint of AMH on testosterone production. The decrease in AMH concentrations has been noted to occur prior to the development of clinical signs of puberty (51); a murine study has showed the pubertal increase in serum testosterone occurred 15 days after AMH concentrations decreased to basal levels (55). Also consistent with a negative correlation with testosterone concentrations, males with delayed puberty exhibit significantly elevated AMH levels, whilst males experiencing a precocious puberty have serum AMH concentrations substantially lower than those of age-matched peers (53, 56). A recent murine study has reported strong inhibitory action of testosterone and DHT on the AMH promoter (57) raising the possibility of a synergistic relationship between testosterone and AMH in males.

Data currently available based on *in vitro* cell cultures and *in vivo* animal studies indicates a strong inhibitory action of AMH on aromatase and 17α-hydroxylase/17,20-lyase, signifying that monitoring of testosterone and oestrogen concentrations would be indicated in phase I trials if AMH was administered to humans in supra-physiological amounts.

THE ROLE OF AMH IN OVARIAN FOLLICLE DYNAMICS

An accelerated rate of primordial follicle recruitment with premature depletion of the ovarian reserve occurs in mice engineered to be homozygous or heterozygous for an inactivating mutation of the AMH gene (58–61). In one study, it was reported that prior to puberty (25 days of age), primordial follicle numbers were comparable between AMH deficient and wild type mice (60). During puberty (four months of age), a more rapid recruitment of primordial follicles was demonstrated, with increased numbers of pre-antral and antral follicles in mice carrying the loss-of-function AMH gene mutation. Towards the end of the reproductive lifespan (thirteen months of age), an almost complete depletion of the primordial follicle pool in mice homozygous for the AMH mutation had occurred, with a lesser reduction exhibited in heterozygous mice. Consistent with a premature exhaustion of the ovarian reserve, a subsequent study reported that 56% of AMH knockout mice had ceased ovulation by 16-17 months of age, compared to 18% of wild-type mice (62).

Short-term *in vitro* cultures of both murine and human ovarian tissue have documented results consistent with AMH acting to restrain primordial follicle development. The ovaries of two-day old mice, when cultured in the presence of AMH were found to contain 59% less primary and secondary follicles compared to control ovaries ($p \leq 0.05$), consistent with AMH restraining primordial follicle recruitment; after four days of culture this difference had widened to 66% ($p \leq 0.05$) (58). To investigate this effect in humans, ovarian tissue obtained from women aged 26-42 years (mean 33.7+/-3.6 years) and was cultured in the presence or absence of AMH. In uncultured control samples, 56% of follicles were observed to be at the primordial follicle stage; after seven days of culture, the proportion of follicles at primordial follicle stage had decreased to 14-26% in specimens cultured with 0-30ng/mL AMH, ($p \leq 0.05$). When cultured in the presence of 100ng/mL, there was no significant difference in the proportion of primordial follicles compared to uncultured ovarian tissue specimens (63).

An extended tissue culture (four weeks) of human ovarian tissue has not replicated this inhibitory action of AMH in primordial follicle recruitment. In uncultured ovarian tissue, 90.5% of follicles were found to be at the primordial follicle stage compared to 54%, 46%, 35% and 43% in samples cultured in control media or media supplemented with testosterone,

AMH or both AMH and testosterone respectively ($p \leq 0.05$ for all groups) (64). The divergent findings are possibly explained by the prolonged period of tissue culture or by the culture media used in this study containing α-MEM, which has been demonstrated to result in significantly greater follicle initiation and growth compared to other culture media (65).

There is a limited amount of data to suggest that AMH may have a supportive role in later stages of follicular growth. In one study, pre-antral follicles (140-150μm) were dissected from ovaries obtained from 12 day old rats and were individually cultured in wells containing FSH, AMH or both FSH and AMH (66). After 72 hours, there was no significant growth of follicles cultured in control media. Follicles increased in size by 10μm when cultured in the presence of AMH, by 25μm in the presence of FSH and by 40μm in the presence of both FSH and AMH, suggesting a facilitatory role of AMH on secondary follicle growth.

In a primate study of adult female macaques, AMH was determined to promote the formation of an antrum in growing follicles (67). In this study, secondary follicles (diameter 125-225μm, 2-4 layers of granulosa cells) were isolated and cultured in individual wells for five weeks. AMH or a neutralizing anti-AMH antibody was added to the wells. Follicles developed an antrum earlier when AMH was added to the culture media in weeks 0-3; the addition of the anti-AMH antibody delayed antrum formation. Oestradiol production was markedly low at week 5 in follicles exposed to AMH compared to control follicles, consistent with an inhibitory effect of AMH on oestradiol production.

In the second part of this study, intra-ovarian infusions of either control of 500ng/hour of anti-human AMH antibodies were administered to adult macaques for day 1-4 of the menstrual cycle until the mid-cycle oestradiol peak (67). In the macaques administered anti-AMH antibodies, antrum formation was delayed. Based on this data, the authors have submitted that AMH facilitates the pre-antral to antral development of ovarian follicles in primates.

MECHANISMS OF CHEMOTHERAPEUTIC DAMAGE TO THE OVARIES

Mechanisms by which chemotherapy may cause depletion of the ovarian reserve include a detrimental effect to the stroma or vasculature; direct damage to the oocytes inducing apoptosis; damage to the granulosa cells that comprise the follicles containing the oocytes; or unrestrained activation of primordial follicles with their consequent rapid loss from the ovarian reserve (68). There is evidence supporting each of these hypotheses.

Vascular damage is an established consequence of many chemotherapeutic agents as demonstrated in a trial in which human ovarian tissue was either cultured with doxorubicin for 72 hours, or xenografted into SCID mice that were then treated with doxorubicin. Reduced vascular density after exposure to doxorubicin was reported in both the *in vitro* and *in vivo* studies (69). In a human study, ovarian tissue samples were obtained from 35 women with cancer (mean age 28.7+/- 7.74 years) of whom 17 had been exposed to non-sterilizing chemotherapy prior to laparoscopic harvest of ovarian tissue. In a blinded histopathological examination of specimens, thickening and hyalinization of large stromal vessels, disordered neovacularisation and regions of focal fibrosis was identified in the ovarian cortex specimens obtained from women who had received chemotherapy (70). Concurring with these findings is a study of ovarian tissue biopsies obtained from girls who had successfully completed chemotherapy treatment for acute lymphoblastic leukaemia; narrowed capillaries with irregular lumens and pericapillary stromal fibrosis was observed in these specimens (71). Whilst these studies establish a feasible mechanism in which chemotherapy causes microvascular damage to the ovarian cortex, potentially leading to areas of localized ischaemia and resulting in primordial follicle loss, causality has not been proven.

The hypothesis that damage to the ovarian reserve is due to direct cellular damage to the oocyte is well founded (68). A study in which high dose cyclophosphamide was administered to mice demonstrated disruption to the morphology of the oocyte with the nuclear contents becoming clumped and distorted within 24-72 hours of administration. The surrounding granulosa cells appeared unaffected upon histological analysis. Complete destruction of the oocyte followed, leaving an empty ring of granulosa cells (72). Another study has demonstrated doxorubicin induces double-strand DNA breaks in both oocytes and granulosa cells (69).

One mechanism of action of alkylating agents, such as cyclophosphamide, is to induce abnormal bonds, or cross-links, between DNA bases preventing strand separation required for transcription or translation. Administration of cyclophosphamide to female rats primed with pregnant mare serum gonadotrophin reported an increase in DNA cross-linkage in granulosa cells two hours after administration. Twenty-four post-administration, granulosa cell numbers were depleted by 51% (73), indicating that damage to the ovarian reserve caused by chemotherapy is not confined solely to direct damage to oocytes. Consistent with this finding was a study of female mice administered with cyclophosphamide that used TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) to detect DNA fragmentation and apoptosis which demonstrated a dose dependent increase in apoptosis of the granulosa cells of preantral and antral follicles. This effect did not extend to the granulosa cells of primordial or primary follicles. This finding is likely explained by the higher mitotic index of the granulosa cells of larger follicles, rendering them more sensitive to cyclophosphamide (74).

Another hypothesis regarding the mechanism of chemotherapeutic damage is that a sharp drop in serum oestradiol concentrations occurs upon the commencement of chemotherapy, with the consequent decrease in the negative inhibitory action on the anterior pituitary causing an elevation in serum FSH concentrations. The FSH elevation would be expected to cause an upsurge in the rate of granulosa cell proliferation, increasing the susceptibility of these cells to the anti-proliferative and cytotoxic effects of chemotherapy. With continued follicular destruction and further FSH elevation, more follicles are recruited

and destroyed, eventually resulting in primordial follicle depletion (75). If this theory is correct, medications that inhibit the release of FSH, namely GnRH agonists, administered during chemotherapy should be protective, however studies in this area have shown divergent results (76–79). Additionally, a study of the hormonal sequalae of chemotherapy has not shown a dramatic drop in serum oestradiol concentrations immediately upon the commencement of CMF (cyclophosphamide, methotrexate, 5-fluorouracil) treatment (80). Further arguing against this theory, is that the finding that granulosa cells of primordial follicles do not express FSH receptor mRNA (81), indicating that primordial follicle recruitment must take place independently of FSH.

Despite these flaws, this hypothesis has merit in suggesting that 'burn out' of the primordial follicle reserve may be an important mechanism by which chemotherapy provokes destruction of the ovarian reserve. In a murine study, increasing doses of cyclophosphamide have been shown to cause an upsurge in the ratio of early growing follicles compared to dormant follicles (82). Should a means of restraining primordial follicle activation become available, its use during chemotherapy may be protective to the ovarian reserve.

ABILITY OF AMH TO PROTECT THE OVARIES DURING CHEMOTHERAPY

If AMH is able to restrain primordial follicle activation, it may be able to offer a degree of protection to the ovarian reserve during chemotherapy. To evaluate this hypothesis, carboplatin (80mg/kg IP) or doxorubicin (3mg/kg IP) was administered weekly to tumour (ovarian cancer) bearing Nu/Nu mice. AMH concentrations were maintained through the use of an AAV9-AMH viral vector which had previously been demonstrated to convey a sustained elevation in serum AMH concentrations. Mice were euthanized when tumour-related end points were met. In the presence of the AMH viral vector, primordial follicle counts were reported as being 2.2-fold higher in mice receiving carboplatin and 1.8-fold higher in mice receiving doxorubicin, compared to controls receiving chemotherapy alone. In a subsequent experiment, osmotic pumps containing an rAMH solution were implanted in mice. Weekly chemotherapy with carboplatin, doxorubicin or cyclophosphamide was then instituted, with the euthanized after two weeks of chemotherapy. Primordial follicle counts were higher in the mice implanted with the rAMH osmotic pumps (1.4-fold higher for carboplatin-treated mice, $p<0.0001$; 2.9-fold higher in doxorubicin-treated mice, $p<0.001$; 1.2-fold higher in cyclophosphamide-treated mice, $p<0.05$) compared to mice implanted with saline pumps (48).

Another study involved the administration of a single intraperitoneal injection of either vehicle, 150mg/kg cyclophosphamide, 5mg/kg rAMH or both cyclophosphamide and rAMH to female mice before being euthanized either 17 hours or 8 days later. The primordial follicle count was reduced in the cyclophosphamide treated mice compared to the control mice (448.2+/-55.0 versus 1197+/-138.2 follicles, $p<0.01$), whereas the number of primordial follicles in the ovaries of mice receiving both cyclophosphamide and rAMH was similar to that of controls (813.4+/-68.7 versus 875.5+/-83.9, p=NS) (83).

The protective effect of AMH on the ovarian reserve during chemotherapy has been demonstrated to translate into an improved fertility outcome in one of two studies. The authors of the study demonstrating a protective effect of AMH (84) administered two doses of cyclophosphamide (150mg/kg) to mice either in isolation or in conjunction with rAMH (four doses of rAMH administered six hourly following each dose of cyclophosphamide). Mice were then mated with males of proven fertility six successive times commencing five weeks after the final dose of chemotherapy. Mice exposed to cyclophosphamide alone demonstrated a reduced pregnancy rate and a smaller mean litter size compared to controls (pregnancy rate: 0.26+/-0.07 versus 0.50+/-0.06, $p<0.05$; mean litter size 1.2 +/-0.3 versus 3.0+/-0.6). Mice exposed to chemotherapy plus AMH had a similar pregnancy rate to controls (0.54+/-0.06 versus 0.50+/-0.06), with a higher mean litter size compared to mice that received cyclophosphamide alone (2.3+/-0.4 versus 1.2+/-0.3).

The second study (83) involved the weekly intraperitoneal administration of either vehicle or a non-sterilizing dose (75mg/kg) of cyclophosphamide for four weeks, either in isolation or in combination with rAMH. Four weeks after the final injection, mating with males of proven fertility took place. The authors reported a non-significant reduction in the cumulative number of mice born in the group receiving cyclophosphamide alone compared to control mice and mice receiving both cyclophosphamide and rAMH (47.3+/-5.9 versus 53.2+/-8.2 versus 53.5+/-10.5). The lack of statistical effect demonstrated may be due to the lower dose of cyclophosphamide (75mg/kg) used in this study, compared to dose (150mg/kg) used in the study that demonstrated a protective effect on litter size with AMH administration.

The administration of AMH during chemotherapy must be conducted with care, avoiding the abrupt withdrawal of exogenous AMH. In a normally functioning ovary, follicles at all stages (primordial, primary, secondary, antral) coexist at the same time. When high levels of exogenous AMH are administered, there is a marked reduction in the number of primary, secondary and antral follicles (48). As the granulosa cells of the secondary, pre-antral and early antral follicles secrete AMH (85), a drop in these follicle numbers would be expected to make the ovary unable to continue to secrete AMH should the administration of exogenous AMH be abruptly ceased. This notion is supported by an observation that immediately post AMH cessation, there is a large and rapid loss of primordial follicles (48).

ROLE IN OVARIAN TISSUE TRANSPLANTATION

The potential use of AMH in respect to fertility preservation is not limited to its administration during chemotherapy. In pre-pubertal girls, or women for whom oocyte cryopreservation is not an option prior to the instigation of gonadotoxic treatment,

ovarian tissue may be cryopreserved prior to the instigation of treatment, with regrafting of the ovarian tissue after successful cancer therapy. Whilst over 130 live births have occurred as a result of this technology (86), there is a massive follicular loss immediately post-transplantation secondary to ischaemia and rapid primordial follicle activation (87–89). This has resulted in a high denominator of transplantation attempts to achieve the reported number of live births. One case series of 111 women undergoing transplantation of cryopreserved ovarian cortex reported that 32 women (29%) conceived and 23 women (21%) had a live birth (a total of 33 live births, with five women delivering more than once and two twin deliveries) (90). Another case series of 95 orthotopic transplantations in 74 women, reported that 16 women conceived (17%) of these women, 15 continued to a live birth (16%) (a total of 17 live births with two women delivering twice) (91).

The transplantation of ovarian cortical tissue into women who have undergone sterilizing cancer treatment (and thus have no ovarian function) introduces the ovarian tissue into an environment devoid of AMH. Parallels can be drawn to the culture of ovarian cortical tissue in media that does not contain AMH; *in vitro* studies consistently demonstrate rapid primordial follicle activation in this situation. One study that utilized ovarian cortex sourced from cattle and baboons demonstrated that the majority or primordial follicles were activated within 12-24 hours of culture in AMH-free media (92). In a study of bovine ovarian cortex, 72% of follicles in freshly harvested tissue were at the primordial stage of development, within two days of culture only 10% remained at the primordial follicle stage (93). In a quantitative analysis of ovarian tissue specimens obtained from women aged 25-42 years (mean age 35 years) who underwent oophorectomy, 88% of follicles were reported to be at the primordial stage, with 8% at the primary stage when examined immediately post-oophorectomy; after 4-11 days of culture, only 20% of follicles were reported to be at the primordial follicle stage, with 65% at the primary stage after 4-11 days (94).

The results of an *in vivo* studies in which human ovarian tissue was grafted into ovarectomized (SCID) mice are consistent with the *in vitro* studies. The proportion of primordial follicles present in the ovarian tissue fell from 72.88+/-5.93% in pre-graft control specimens to 38.95+/-3.94% four weeks after grafting ($p<0.001$) and 37.42+/-5.83% ($p=0.009$) twelve weeks after grafting. There was a concomitant rise in the percentage of primary follicles from 13.48+/-2.92% in pre-graft control specimens, to 29.29+/-2.60% four weeks after grafting ($p=0.009$), but twelve weeks after grafting the elevation in primary follicle percentages was not sustained, with 9.74+/-2.22% of follicles found to be at the primary follicle stage. This latter result is likely due to a substantial number of the primary follicles progressing to the secondary stage [pre-graft versus four week graft 13.37+/-6.09% versus 27.60+/-4.16% ($p=0.018$); pre-graft versus twelve week graft 13.37+/-6.09% versus 49.40+/-6.14 ($p=0.001$)] (95). These results concur with a similar study that reported 71% of follicles were at the primordial stage in fresh human ovarian cortex; one week after grafting into nude mice, this proportion had decreased to 39% ($p<0.001$); a concurrent rise in the proportion of primary follicles from 11% in fresh tissue to 24% stage one week after grafting was observed ($p=0.05$) (87).

The mass activation of primordial follicles that occurs in the absence of AMH is in contrast to the orderly activation of primordial follicles that occurs in the ovaries of healthy women. A key difference between these two situations is that AMH is present in the circulation of the healthy women. The pivotal role that AMH has in controlling primordial follicle activation was demonstrated in a study in which human ovarian cortex was cultured in the presence or absence of AMH. Rapid depletion of the primordial follicle pool occurred in AMH-free media, however when 100ng/mL AMH was added to the media, the proportion of follicles maintained at the primordial stage was comparable to that of uncultured tissue (63). Another study using bovine ovarian cortex demonstrated that when this tissue was cultured in serum-free media, a seven-fold decrease in primordial follicles occurred. When bovine ovarian cortex was cultured on the edge of the chorioallantoic membrane (CAM) of chick embryos, primordial follicle numbers were unchanged from day 0, indicating a component in the environment of the ovarian tissue was able to restrain the activation of primordial follicles (92).

The clinical experience of human auto-transplantation of cryopreserved ovarian cortex is consistent with unrestrained primordial follicle activation. In a longitudinal analysis of eleven young menopausal recipients of fresh ovarian cortex transplants (donated by an identical twin, n=9, or a non-identical sibling, n=2) AMH concentrations initially remained low before sharply rising to supra-physiological levels (at approximately 170 days post grafting), consistent with a rapid progression of primordial follicles to the secondary stage whereupon AMH secretion is commenced. Shortly thereafter, a sustained fall to below to sub-physiological concentrations was observed (approximately 240 days post grafting), consistent with a decrease in secondary follicles secondary to depletion of the primordial follicle pool (96).

It is conceivable that the administration of exogeneous AMH could temper the rapid initial recruitment and subsequent loss of primordial follicles until reperfusion of the graft has taken place and intrinsic AMH production commenced. To elucidate the effect of AMH on ovarian tissue, researchers cryopreserved murine ovarian tissue in vitrification media containing different concentrations of human AMH (0, 5, 15 or 45μg/mL) before warming the tissue in media using the same concentrations of AMH. No difference was detected in the proportion of primordial, growing or grade 1 follicles between the different dose groups of AMH, although there was a reduction in the proportion of apoptotic follicles from 21.0% in the control group compared to 8.1% in the 5μg/mL AMH group and 1.7% in the 15μg/mL and 45μg/mL AMH groups (97). The lack of difference in the proportion of follicles at different stages may be explained by short duration (total 35 minutes) of exposure of the ovarian tissue to AMH.

The same researchers then vitrified fresh ovarian cortex in media devoid of AMH, before warming and auto-transplanting the tissue back into the mice one week later. The mice were

divided into groups and received either 0, 50, 250 or 1,250μg/mL human AMH doses every two days for a total of four doses prior to ovariectomy, immediately post ovariectomy or both pre- and post-ovariectomy. Mice were euthanized either 7 or 28 days after auto-transplantation. No statistical difference in the proportion of primordial or growing follicles was identified in any of the treatment groups (97). The absence of effect in the second study may be explained by an insufficient frequency of AMH. The decision of the authors to administer AMH every two days was based on a report estimating the half-life of bovine AMH to be approximately 48 hours (98), however a study of human AMH has estimated the half-life as 27.6 hours (99).

Further *in vivo* research using human ovarian cortex is required to clarify whether AMH has the capacity to curtail uncontrolled mass activation of primordial follicles in the immediate post-transplantation period. The prolonged timeframe between fresh ovarian tissue grafting in young menopausal women and rise in AMH (96), suggests an extended duration of AMH administration may be necessary.

ROLE OF AMH IN THE TREATMENT OF CANCER

As its name suggests, the renowned physiological function of AMH is to induce the regression of the Müllerian ducts in the male fetus. It has been hypothesized, and successfully demonstrated, that this inhibitory action can be utilized to induce the regression of malignancies expressing AMHRII (100). Studies have demonstrated that AMH is capable of inhibiting the proliferation of cancers arising from Mullerian structures such as the cervix (101, 102) and endometrium (103, 104), as well as some cancers arising from non-Mullerian structures that express AMHR II such as breast (105–107), vulva (108) and prostate (106). The main focus of investigation however has been in regard to ovarian cancer. Despite the ovaries not being Mullerian structures, there is increasing concurrence that many ovarian neoplasms are of Mullerian origin (109–111), explaining why AMHR II receptors are expressed by many ovarian cancer cells (112, 113).

Initial studies used partially purified bovine AMH to demonstrate that AMH was capable of inhibiting growth *in vitro* of human papillary serous cystadenocarcinoma cells (100). *In vivo*, pretreatment of ovarian cancer cells (HOC-21 cell line) with bovine AMH delayed the appearance of tumour and increased disease free survival when these cells were subcutaneously injected into the middorsal flank of Balb/C nude mice (114). Fresh tumour suspensions were obtained from twenty-eight women undergoing surgery for gynaecological cancer (ovarian, endometrial or fallopian tube origin) and were tested in soft agar colony inhibition assays. Significant colony inhibition was demonstrated in 25 of 28 assays following incubation with bovine AMH (115).

After the human AMH gene was isolated, it was transfected into Chinese hamster ovary cells to provide a more purified form of AMH (recombinant AMH, rAMH). *In vitro* studies confirmed that rAMH could inhibit both human and murine ovarian cancer cell lines (112, 113, 116, 117). In one study, six human ovarian cancer cell lines expressing AMHR II were incubated in the presence of 15ug/mL rAMH (113). rAMH caused almost complete inhibition of growth in two cell lines, and significant inhibition of growth in three other lines. In the final cell line colony growth was not inhibited, possibly secondary to impaired downstream signaling due to an absence of functional p16. Ascites was then obtained from 27 women with ovarian cancer of which 15 (56%) contained malignant cells that bound biotinylated AMH, suggesting expression of AMHR II. Out of the eleven cell lines that bound biotin and grew in soft agarose, 9 of 11 (82%) were significantly inhibited (29-94% inhibition) when rAMH was added to the culture medium.

In vivo studies using murine (MOVCAR7, MOVCAR8) and human (OVCAR3, OVCAR8, IGROV-1) cancer cell lines injected into immunodeficient mice have also demonstrated an inhibitory effect of rAMH (108, 117, 118). Additionally, rAMH may have anti-metastatic action, having been shown to decrease invasiveness in an *in vitro* study using the epithelial ovarian cancer cell line IGROV-1, and inhibit migration in an *in vivo* study using a chick chorioallantoic membrane migration assay (119).

Epithelial ovarian cancer is a highly lethal cancer due to an advanced disease stage at the time of diagnosis in the majority of cases and chemo-resistance that may be intrinsic or develop with disease progression (120). High grade ovarian cancers exhibit biological features, including molecular heterogeneity, the capacity to metastasize and an ability to develop chemo-resistance, that support the proposal that it is a cancer stem cell driven disease. Cancer stem cells are capable of unlimited self-renewal and have an ability to differentiate through asymmetric cell division. As they are pluripotent, they can give rise to daughter cells with different phenotypes, permitting tumour heterogeneity and chemo-resistance (121). Their relatively quiescent state and expression of proteins capable of acting as molecular pumps, effluxing lipophilic medications out of the cell, permit these cells to evade destruction by chemotherapeutic and radiation treatments (121, 122).

As AMH acts by binding to the extracellular AMHR II rather than by diffusing into cells, it has the capacity to act on chemo-resistant cancer stem cells. An *in vitro* study demonstrated that whilst treatment of cells from the OVCAR-5 ovarian cancer cell line with doxorubicin, cisplatin and paclitaxel resulted in a decrease in the total number of viable cells, there was an expansion in the proportion of cells exhibiting stem cell characteristics. Treatment with AMH caused a significant decrease in both the total number of cells and in the stem cell population (123). An additional study that isolated stem cell enriched populations of ovarian cancer cell lines concurred that doxorubicin treatment stimulated the growth of these cells, whilst AMH inhibited proliferation by inducing G1 arrest through the induction of cyclin-independent kinase inhibitors (124).

The potential clinical utility of AMH to inhibit the proliferation of chemo-resistant ovarian cancer cells was demonstrated in a

study in which malignant cells were obtained from the ascites of women with highly resistant ovarian cancer. *In vitro*, four of six cell lines were inhibited when cultured in media containing AMH. To investigate *in vivo* action, five patient-derived lethal chemo-resistant serous adenocarcinoma lines were xenografted into mice. AMH administration inhibited the proliferation of three of the five tumours (125).

AMH also has potential utility in immunotherapy-based approaches to cancers expressing AMHR II. A mouse antibody (12G4) has been developed to bind to the human AMHR II. When administered to nude mice xenografted with human granulosa cell (COV434) or epithelial ovarian cancer (OVOCAR-3) cell lines, tumour growth was inhibited *via* antibody-dependent cell-mediated cytotoxicity (possible as nude mice have functional macrophages and natural killer cells) and, to a lesser extent, due to the activation of signaling pathways after receptor/ligand complex internalization (126).

Murlentamab (GM102 or 3C23K) is a human monoclonal antibody designed to bind to the AMHRII expressed by malignant cells. The Fc portion of the antibody has been glycol-engineered to have low fucosylation, enabling high affinity binding to CD16 and thus enhancing natural killer cell activity as well optimizing antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (127, 128). A phase I trial of murlentamab of 68 women with metastatic AMHRII-expressing ovarian, cervical or endometrial cancer who had previously been treated with at least one chemotherapy regime reported that the antibody was well tolerated with no dose limiting toxicity; the most common adverse effect was G 1-2 asthenia (29%); a lesser number of patents (12%) reported more severe asthenia, nausea or vomiting (129). Approximately 80% of colorectal adenocarcinomas express the AMHRII, consequently a phase II study of the efficacy of murlentamab in patients with advanced colorectal adenocarcinoma has been conducted (130). Thirty nine patients received either murlentamab alone (n=14) or in conjunction with trifluridine and tipiracil (n=15), with the study reporting a 1.7-fold and a 3.6-fold reduction in the tumour growth rate in these groups respectively. Of fourteen patients treated with murlentamab alone, those with greater than 20% AMHRII positive tumour cells, showed superior rates of progression-free survival.

Antibodies specific for AMHR II have been radiolabelled with 213Bi to effect destruction of AMHR II expressing intra-peritoneal tumours in mice (131) and can be used as a means of targeted drug delivery whereby cytotoxic drugs are covalently attached to AMH. Upon internalization, the bonds between AMH and the cytotoxic molecule are cleaved by proteases, freeing the drug to accomplish its cytotoxic function (9, 121). The use of radiolabelled diabodies (constructed from fragments of antibodies capable of binding to AMHR II), has been proposed as a new immunoimaging diagnostic and monitoring approach to gynaecologic malignancies (132).

AMH in the treatment of cancer has a number of potential advantages over traditional chemotherapeutic agents. Firstly, increased concentrations of AMH do not convey the severe toxicity associated with many chemotherapeutic medications. AMH concentrations of up to 480ng/mL in women with PCOS (133) and 1,200ng/mL in women with granulosa cell tumours (134) have been reported without evidence of adverse effects. Secondly, AMH acts *via* a different mechanism to traditional chemotherapeutic agents and therefore may be effective in the treatment of cancer stem cells and chemo-resistant cancers. Finally, in contrast to many chemotherapy medications currently used in clinical practice that damage the ovaries and thus reduce the likelihood of future fertility, AMH would not be expected to cause a depletion of the ovarian reserve provided its administration is not abruptly ceased. If AMH was co-administered during chemotherapy, it may have a synchronous action to the chemotherapy, and may also offer a degree of protection to the ovaries.

CONCLUSIONS AND FUTURE DIRECTIONS

Preliminary animal data indicates that AMH has clinical application in protecting the ovarian reserve during chemotherapy, suppressing primordial follicle recruitment whilst vascularization of ovarian tissue grafts is re-established and in the treatment of malignancies expressing AMHR II.

To date, AMH has not been administered to humans. A comprehensive review of the biological actions of AMH suggests that its administration would be safe and well tolerated, although a decrease in sex steroid production would be expected due to its inhibitory of aromatase and 17α-hydroxylase/17,20-lyase. Depending on the degree of suppression, oestradiol supplementation may be required to avoid menopausal symptoms or if AMH administration was prolonged. Administration of an AMHRII antibody (murlentamab) has been well tolerated in a small number of cancer patients (129, 130).

Epithelial ovarian cancer accounts for approximately 90% of ovarian cancer diagnoses, of which the majority have serous tumour cell histology. 80% of serous epithelial ovarian cancers are diagnosed at an advanced (III-IV) stage. The overall five year life expectancy of epithelial ovarian cancer in the USA in 2007-13 was 41% for women diagnosed at stage III and 20% for women diagnosed at stage IV (135) as chemo-resistance inevitable occurs leaving few treatment options aside from palliation. In light of these grim statistics, human trials of AMH administration in women with advanced ovarian cancer would be appropriate, especially data suggests that AMH can be effective in inducing the regression of chemo-resistant tumour cells.

Additional animal studies are required to confirm and detail the value of AMH in fertility preservation agent, both in its capacity to protect the ovarian reserve during chemotherapy and in the context of ovarian tissue grafting after successful cancer treatment. If data consistently confirms the beneficial denoted in preliminary studies, a trial of adjuvant AMH during chemotherapy in women of reproductive age would be pertinent.

As the administration of AMH would be expected to suppress primordial follicle recruitment and development, extended

administration of AMH is likely to decrease the number of pre-antral follicles present in the ovaries, with a consequent decrease in intrinsic AMH secretion. Abrupt cessation of AMH administration would therefore be expected to result in a transient period of sub-physiological AMH serum concentrations and uncontrolled primordial follicle recruitment could occur. This mandates caution with use of AMH for the purpose of preserving the ovarian reserve during chemotherapy; graduated dose reduction prior to cessation is warranted.

AUTHOR CONTRIBUTIONS

RR conceived and designed the article, conducted the review of the literature, interpreted the data and wrote the manuscript. JA contributed to data analysis and conducted a critical revision of the article. KW conducted a critical revision of the article. WL contributed to data analysis and conducted a critical revision of the article. All authors contributed to the article and approved the submitted version.

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