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Chapter 15

**A CRITICAL EVALUATION OF WHETHER
CIRCULATING ANTI-MÜLLERIAN HORMONE IS A
HORMONE IN ADULTS, WITH SPECIAL REFERENCE
TO ITS PUTATIVE ROLES IN MEN**

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ABSTRACT

Anti-Müllerian hormone (AMH) is present in blood but despite its nomenclature has no overt endocrine roles. This chapter reviews the basic endocrinology of AMH, and discusses why its functions might be cryptic. AMH is a member of the TGF β superfamily that produce context-dependent regulation of development and homeostasis. This large family share a small number of receptors and binding proteins resulting in cellular signalling that emerges from multiple interactions. Consequently, the absence of one ligand typically results in subtle dysregulation, rather than an overt loss of function. AMH has been considered to be an atypical TGF β , as it uniquely triggers the degeneration of the Müllerian duct. We suggest that AMH displays distinct paracrine and endocrine signalling. Systemic AMH may be a conventional TGF β superfamily ligand that exerts a gonadal influence to TGF β signalling in multiple tissues. Circulating AMH is a mixture of the uncleaved prohormone (proAMH) and a non-covalent complex of the N- and C-terminal peptide (AMH_{N,C}). ProAMH cannot activate AMH receptors, and the AMH-mediated gonadal influence may be modulated by the regulation of its cleavage, both within and outside of the gonads. Ovarian AMH exhibit age-dependent decline, but the age-related changes in testicular secretion are more variable. Elderly men on average

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have lower levels of AMH than younger men, but with high intrapersonal variation. Some elderly men lack AMH in their circulation. These age-related changes in AMH are only weakly linked to the other Sertoli cell hormone (inhibin B) and the leydig cell hormones. Consequently, elderly men are very diverse in their testicular output. The examination of the traits of men with varying levels of AMH is at an early stage, with initial studies showing multiple associations between AMH and cardiovascular parameters. In conclusion, AMH appears to be a non-classical hormone whose functions have been hidden because it is a component of a complex regulatory system. The importance of AMH and the breadth of its functions are largely unknown.

INTRODUCTION

Anti-Müllerian hormone (AMH) is present in the circulation, and its name proclaims it to be a hormone. Despite this, there is no consensus that AMH is a hormone, particularly in adults. In this chapter, we critically review the evidence for and against circulating AMH having a physiological function in adults, with particular reference to its putative functions in men.

The discovery of AMH dates from an era when the classical hormones were first investigated [1, 2]. *AMH*^{-/-} humans have been described [3] and *Amh*^{-/-} mice generated [4]. Hence, AMH is most unlikely to conform to the norms of classical hormones, for if it did, its endocrine functions would have been described long ago. One of the objectives of this chapter is therefore to identify aspects of the biology of AMH which would result in its endocrine actions being cryptic.

This chapter is divided into several sections, each of which examines a distinct aspect of the basic endocrinology of AMH. By definition, a hormone must be present in blood, and this chapter begins by reviewing recent evidence about the forms of AMH in blood, followed by a discussion of where the AMH in circulation may be derived from. The target cells of hormones are defined by the location of their receptors. The second section therefore discusses what the receptors for circulating AMH may be, and what the biological consequence of activation of the receptors might be. The third and last section then reviews the emerging evidence relating to the putative physiological functions of circulating AMH.

1. ORIGINS AND FORMS OF CIRCULATING AMH

Circulating AMH Is Gonadal

In all species studied to date, the main site of AMH production is the gonads. During development, the gonadal production of AMH is high and male specific, with a lower and less dimorphic expression emerging during the pubertal transition [5, 6]. The gonads are normally the sole source of AMH in the blood, as boys with anorchia lack AMH [6] and as serum AMH diminishes to trace levels after either orchidectomy [7] or oophorectomy [8, 9]. This lays the foundation for the use of serum AMH levels as a biomarker of gonadal function.

AMH May Also Have Conserved Paracrine Functions

Low levels of AMH mRNA have been detected in the ventricular myocardium of human neonates [10] and adult murine brains [11, 12]. AMH production also occurs at these sites in some fish [13, 14], indicating that the pattern of gonadal and non-gonadal production of AMH predates the evolution of the Müllerian duct. This degree of conservation strongly suggests that AMH has unknown functions that are critical to fitness. Additionally, mRNA for AMH and its receptors have been detected in endometrial [15] and endometriosis cells [16], suggesting a possible paracrine role for AMH in the uterus [15].

The probable co-existence of paracrine and endocrine roles for AMH complicates the detection of the endocrine functions of AMH, as the cellular location of AMH receptors will not unambiguously identify the target cells of circulating AMH. It also means that part of the phenotype of AMH^{-/-} individuals may be unrelated to the functions of gonadal AMH.

AMH Has Multiple Forms

AMH is synthesised as a 560 amino acid preproprotein homodimer [17]. The 24 amino signal peptide [17] is thought to be removed intracellularly, giving rise to an inactive propeptide (proAMH) [18-20]. ProAMH can be enzymatically cleaved to generate N-terminal (AMH_N) and C-terminal (AMH_C) peptides, which form a stable non-covalent complex (AMH_{N,C}) (Figure 1). AMH_{N,C} and AMH_C are both receptor competent, but free AMH_C has not been detected in biological fluids [21] and may only be generated *in vivo* as AMH_{N,C} dissociates during receptor activation [20]. Recombinant proAMH is also cleaved inefficiently at a second site to yield a larger C-terminal peptide (AMH₂₂₅₋₅₆₀) [21, 22]. This may correspond to the ancestral form of AMH in fish, which utilise a larger monomeric form of AMH_C [23, 24]. AMH₂₂₅₋₅₆₀ has not been detected in the serum of healthy humans to date [21], but its presence in the serum of a patient with a sex cord tumour [22] and equine granulosa cell tumours [25], raises the possibility that serum AMH₂₂₅₋₅₆₀ may have utility for detecting and monitoring AMH-producing tumours.

ProAMH Is Present in Blood

Human blood contains a mixture of proAMH and AMH_{N,C} [21]. This unexpected observation begs multiple questions that are central to the understanding of AMH as a hormone. For example, why do the gonads release inactive AMH, and does the proportion of cleaved AMH vary with physiological rhythms? Is the proAMH in blood cleaved outside of the gonads? If so, is the cleavage undertaken by the target cells or some other cell type/organ?. Theoretically, the latter would result in an active hormone whose levels are the product of both gonadal and non-gonadal influences.

The current generation of AMH ELISA provide an aggregate measure of proAMH and AMH_{N,C} [26], and are not suitable for answering the above questions. We are therefore developing proAMH-specific and AMH_{N,C}-specific immunoassays [27] to permit direct measurements of the AMH species in the circulation and other fluids. In the interim, a

preliminary answer to these questions can be obtained by considering which enzymes cleave proAMH.

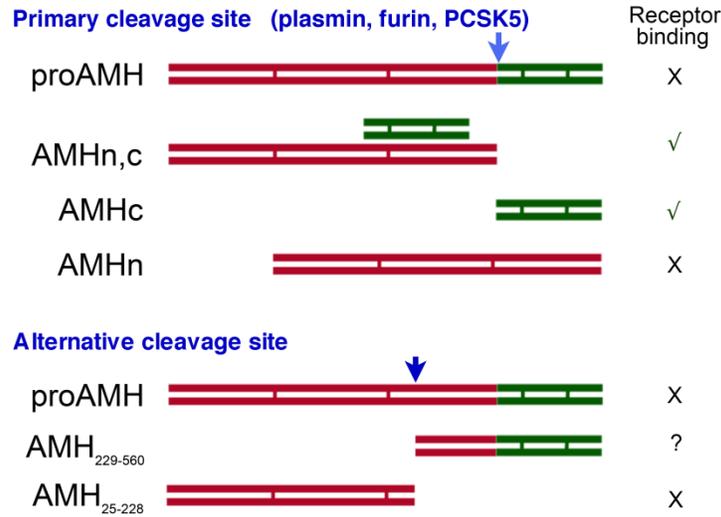


Figure 1. Schematic illustration of the forms of AMH. ProAMH and AMH_{N,C} are present in the circulation of the boys and adults [21].

Multiple Enzymes May Cleave proAMH

Recombinant AMH is cleaved by multiple members of the subtilisin/kexin family of prohormone convertases, most notably PCSK3 (furin) and PCSK5 [28]. PCSK3 and 5 are produced by Sertoli cells, and are putatively responsible for the testicular production of AMH_{N,C} [28], although this has not been proven. PCSKs are also expressed within the ovary [29, 30], with PCSK5 being upregulated during murine follicular development [31], possibly under the control of gonadotropins [32]. Many non-gonadal cell types also synthesise relevant PCSKs, including neurons and aortic cells [33-36]. The expressions of these enzymes are highly regulated, and vary in response to physiological and pathological stimuli [34, 35, 37].

ProAMH is also cleaved *in vitro* by plasmin [19, 22]. Plasmin is a serine proteinase that cleaves the proforms of cytokines, in addition to its hallmark function of dissolving fibrin blood clots. It is synthesised as a larger precursor, plasminogen, which is activated by various proteases (plasminogen activators, PAs), whose activities are regulated via activators and inhibitors [38, 39]. Multiple cell types in the testes secrete either urokinase-type PA and/or tissue-type PA, including the Sertoli cells, Leydig cells and spermatocytes, in a stage dependent manner [40]. The synthesis of PAs by rodent Sertoli cells is subject to complex paracrine regulation [40], and increases markedly at puberty [41], putatively under the influence of testosterone [41]. In men, the levels of PA vary during the seminiferous cycle, with the highest levels being observed at stages VII-VIII [41]. Inhibitors of PAs are also synthesised within the testes [42], further indicating the complexity by which this enzyme is regulated in the gonads.

Plasminogen and PAs are also present in ovarian follicular fluid [43], with the generation of active plasmin by granulosa cells being under the control of gonadotrophins [44, 45]. This plasmin has been implicated in the development and rupture of the follicular wall at ovulation [46], with low plasmin activation being implicated in the pathogenesis of polycystic ovary syndrome [47].

Summary

The available evidence suggests that the cleavage of proAMH within the gonads is highly regulated. Thus, although proAMH is a product of Sertoli or granulosa cells, the amount of proAMH and AMH_{N,C} secreted into blood may reflect the broader physiological state of the gonad. ProAMH is almost certainly cleaved outside of the gonads, but it is unknown whether the resulting AMH_{N,C} returns to the circulation and/or is catabolised within tissues. The origin(s) of circulating AMH_{N,C} are therefore currently unclear.

2. WHAT ARE THE RECEPTORS FOR CIRCULATING AMH?

The embryonic and neonatal brain is one of the putative targets of circulating AMH in immature males [48-50]. Embryonic motor neurons *in vitro* respond to AMH with a log-linear dose curve [11]. Consequently, AMH produces significant neuronal survival at both adult-like (20-50 pM) and at boy-like (0.5-1.5 nM) levels [11].

This strongly supports the hypothesis that the AMH in the circulation is bioactive. However, this conclusion is inconsistent with the prevailing model of AMH signalling (below), which is based on the study of gonadal cells and the regression of the Müllerian duct. This suggests that AMH has two modes of signalling: a paracrine mode that requires high levels of AMH and an endocrine mode that functions at lower concentrations.

AMH is a member of the TGF β superfamily that signals through complexes consisting of type 1 and 2 receptors, with both receptors being required for signalling to occur [51]. AMH is the only member of the family that has a unique receptor, AMHR2 [51]. Mutations of AMHR2 lead to a clinical phenotype that mirrors AMH deficiency, illustrating the importance of this receptor for the classical actions of AMH [3, 52].

Endocrine Signalling May Be Initiated by a Binding Protein

Canonically, AMH binds to AMHR2, leading to recruitment of a type 1 receptor, which activates the intracellular cascade. However, the K_d of AMHR2 for AMH is 2-3 nM [53, 54]. Consequently, circulating AMH cannot signal through this mechanism, as the concentration of AMH in blood is insufficient to produce significant activation of AMHR2. AMHR2, however, appears to be required for the endocrine actions of AMH, as the AMH-dependent male biases in the size of brain nuclei are absent in AMHR2^{-/-} mice [48].

The dose response curves of some TGF β superfamily ligands are modulated by binding proteins, with each of the binding proteins influencing a distinct subset of the ligands [51].

We are therefore screening known TGF β superfamily binding proteins to determine whether any modulate AMH signalling. To date, two putative binding proteins have been identified, follistatin 288 and 315 (FS288, FS315), both of which amplify AMH signalling in a reporter assay [55]. The influence of the follistatins is dependent on the concentration of AMH, with the largest effects occurring when AMH is present at serum levels (pM). When AMH is present at nM concentrations, the follistatins have little or no effect, suggesting that they do not modulate paracrine AMH signalling [55].

TGF β Superfamily Signalling Is Contextual

The TGF β superfamily contains over 30 human cytokines, which share five type 2 and seven type 1 receptors [51, 56]. The ligand specificity is the product of both type 1 and 2 receptors, but the intracellular cascade is induced by type 1 receptor. The type 1 receptors activate both canonical and non-canonical pathways, with the main canonical pathways converging into two streams: the SMAD1/5/8 or SMAD2/3 pathways. Consequently, the TGF β superfamily ligands are not independent regulators. They are part of a regulatory mechanism that is integrating multiple influences on a cell, with the integrated influences then eliciting a cellular event that is largely determined by the history of the cell [51, 56, 57].

Consequently, the TGF β s can induce or inhibit apoptosis or cell division or some other cell behaviour in the same cell type in a context dependent manner [56, 57]. This is particularly relevant to the understanding of the gonads, where the molecular context of cells change during the ovarian and seminiferous cycles.

AMH May Be a Typical TGF β Ligand

We suggest that AMH is a typical TGF β superfamily ligand, and that the understanding of its biology has been retarded by the concept that it is unique and different. AMH uniquely has a ligand-specific type 2 receptor, but its type I receptors (ALK2, 3 and 6 [58]) are shared with other TGF β superfamily ligands, most notably the bone morphogenetic proteins (BMPs) and the growth and differentiation factors (GDFs) [51]. Consequently, when AMH activates a cell it is activating a pathway (SMAD1/5/8) that is common to at least 19 other cytokines (Figure 2).

The TGF β superfamily ligands can signal as independent regulators, provided the pattern of expression of the ligands, receptors and binding proteins results in a SMAD pathway being predominantly or solely controlled by a single ligand. Consequently, mice (or people) with null mutations of TGF β superfamily ligands have overt phenotypes, which tend to be restricted to specific tissues at specific stages of development/life cycle. In this context, the persistence of the Müllerian duct in AMH^{-/-} males is not evidence that AMH is an atypical TGF β , with a single function. The various effects of pleiotropic regulators are often a mixture of the overt and the cryptic.

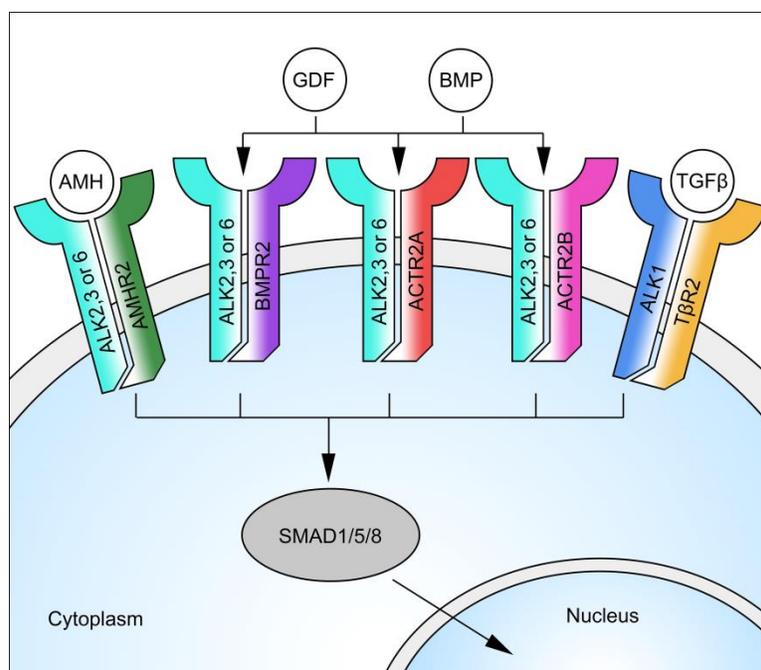


Figure 2. Schematic illustration of the relationship of AMH signalling to that of other members of the TGFβ superfamily. The human bone morphogenetic proteins (BMP2, 4-7, 8A, 8B, 9, 10), growth and differentiation factors (GDF5-7, 9b, 10, 11, 15) and a proportion of transforming growth factors (TGFβ1-3) activate the SMAD1/5/8 pathway, which increases the expression of various genes, depending on a cell's history and current context [51, 56, 57].

AMH Receptors May Be Broadly Expressed

The levels of AMHR2 are somewhat paradoxical, which initially obscured the distribution of this receptor. AMHR2 is one of the most abundant cytokine receptors in neurons, yet the abundance of AMHR2 in neurons is two orders of magnitude less than in the gonads and Müllerian duct [11]. The reasons why AMHR2 is so abundant in the gonads is unknown, although it reinforces the hypothesis that the signalling mechanisms for local/paracrine and circulating AMH are not identical. If the gonads are used as a positive control for AMHR2, to set the sensitivity of detection assays, then the lower levels of AMHR2 in the endocrine target cells will not be detected. AMHR2 mRNA can be detected by qPCR in most tissues, suggesting that it is broadly expressed. However, the detection of AMHR2 protein by immunohistochemistry has proved to be difficult as many commercial antibodies cross-react with other proteins, in a tissue-specific manner. Moving forward, it is important that AMHR2^{-/-} controls are used to prove the cellular locations of AMHR2 in any novel study.

It is premature to discuss the location of AMH binding proteins, as the proof of their identity is at a too early stage. However, it is worth briefly noting that FS315 is a serum-specific protein [59], which may clear activins from the circulation, by facilitating their binding to the cell surface [60]. FS288 is broadly expressed and binds to the heparan-sulphate proteoglycans on cell surface, where they sequester activins [60]. The activins are TGFβ

superfamily ligand, which activate the SMAD2/3 pathway. This raises the intriguing possibility that the follistatins influence the balance between the two TGF β superfamily pathways (SMAD1/5/8 vs SMAD2/3), by enhancing circulating AMH and inhibiting circulating activin activity. This emphasises the fact that the elucidation of context dependent signalling requires a holistic approach in which the presence or absence of all relevant components is known and considered.

Summary

AMH is a TGF β superfamily ligand, and circulating AMH may be one component of a complex regulatory system that involves interactions between TGF β superfamily ligands, receptors and binding proteins. The understanding of how circulating AMH may contribute to this mechanism is limited by the paucity of information about AMH binding proteins, and because the cellular locations of AMHR2 have not been systematically described.

3. PUTATIVE PHYSIOLOGICAL ACTIONS OF CIRCULATING AMH IN MEN

If circulating AMH is one of many cytokines that collectively control the SMAD1/5/8 pathway, then the traits affected by it may be evident when a person's level of AMH is correlated against his/her traits. This includes the propensity to develop a particular clinical condition. The theoretical proviso here is that the TGF β signalling pathway is not able to fully compensate for the loss of AMH.

In complex systems, defects are sometimes more overt in the elderly, for two reasons. First, poorly regulated systems can be relative normal in the short term, but be prone to accelerated degradation over time. Second, homeostasis becomes progressively less precise as redundant "backup" systems degrade. Theoretically, this may result in individuals with naturally low or high levels of AMH beginning to exhibit clinical symptoms as they become elderly. We have therefore been interested in correlating the traits of older men with their levels of AMH. The first step in this process was to describe the range of AMH values in a population drawn from the community.

Circulatory AMH Levels in the Elderly

During ageing, the plasma levels of AMH become progressively dimorphic. Women begin to lose AMH in their mid-twenties [61], with post-menopausal women having little or no AMH in their blood [62]. Men, in contrast, have a much slower decline in AMH levels, beginning in their forties but with high inter-person variation (Figure 3) [6, 63]. A minority of men aged over 70 years have undetectable levels of AMH, whereas other elderly men have levels that lie within the upper range for young men [63]. Moving forward, it will be interesting to determine whether the few elderly men who lack AMH in circulation tend to have a higher incidence of conditions which have a female bias.

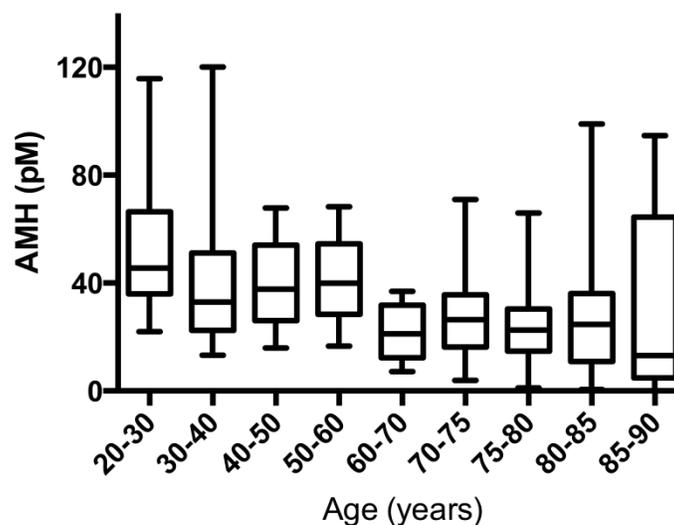


Figure 3. Age related distribution of serum AMH values in a population of 210 community-dwelling men located in Dunedin, New Zealand. The majority of the men were recruited as part of a previous study [63], with their AMH levels remeasured using the Beckman Coulter, ELISA (A79765), in accordance with field safety notice FSN-20434-3, June 2013. A minority of young men have higher AMH values, which is in accord with previous data [5], which may reflect the last stages of transition from the higher boy levels. The data is the median and 5-95 percentile.

The Gonads Release Multiple Hormones

AMH has two contexts, both of which may be pivotal to understanding its function. Its context for signalling is the TGF β superfamily (above). For its synthesis, the context is the gonads. The testes contain two endocrine cell types, the Sertoli and Leydig cells, both of which release two hormones. This creates the possibility that AMH levels are partially coupled with the other Sertoli cell hormone (Inhibin B, InhB), with their levels being influenced by the androgens or insulin-like peptide 3 (INSL3) from Leydig cells. The presence or absence of associations between AMH and other testicular hormones will partially define the physiology of AMH. Knowledge of these associations is also important when interpreting correlative studies between AMH and traits.

AMH and InhB Are Distinct Biomarkers of Sertoli Cells

The levels of the two Sertoli cell hormones weakly correlate in young men ($R^2=0.23$), indicating that AMH and InhB vary independently of each other whilst at the same time being bound by a common influence. Consequently, the ratio of the AMH to InhB levels in men varies within a relative narrow range, even though the absolute levels of both hormones are highly variable between men [63].

Sertoli cells are lost during ageing [64, 65]. Consistent with this, the mean levels of AMH and InhB in serum are lower in older than younger men [63]. However, this is not the only age related change, as the range of ratio of AMH to InhB increases, at both extremes of the

range [63]. This indicates that the Sertoli cells of some elderly men preferentially decrease their release of AMH or InhB and/or preferentially increasing the release of AMH or InhB. Hence, the age-related changes in Sertoli cells encompass dysregulation/dysfunction as well as cell loss [63].

The Sertoli cells of boys produce very high levels of AMH and sub-adult levels of InhB, with boys therefore invariably having a very high AMH to InhB ratio [5, 6, 66, 67]. To date, no elderly men has a boy-like AMH to InhB ratio, indicating that hormonal perturbations of Sertoli cells does not involve dedifferentiation to an immature phenotype.

Age-Related Hypogonadism

The clinical diagnosis of hypogonadism is defined by an insufficiency of testosterone. However, the Leydig cells are part of the same organ as Sertoli cells, and there is a complex inter-relationship between Leydig and Sertoli cells, both during development and in the adult. This begs the question of whether age-related changes in the testes are specific to the hormone, endocrine cell type and/or is pan testicular?

The preliminary data from our Dunedin cohort suggest that the Leydig and Sertoli cell hormones of individuals only weakly correlate, indicating that each of the levels of the four gonadal hormones vary independently of each other. The levels of the hormones become more highly correlated with age, indicating that generalised deterioration of the testes is occurring during ageing, although the extent of this is moderate in most individuals: in most elderly men, the levels of the four testicular hormones are not associated. Consequently, a minority of men are deficient in all four hormones, with a greater number being deficient in only or two of the testicular hormones. Men with low levels of testosterone can be asymptomatic or exhibit a range of diverse symptoms, for unknown reasons [68, 69]. One obvious issue here is whether the diversity in symptoms of men with low testosterone is causally due to the variation in their levels of the other gonadal protein hormones, including AMH.

The partial linkage between the four testicular hormones noted above may confound correlative analysis of these hormones. Moving forward, we argue that all four gonadal hormones should be measured in research studies of gonadal function, with the relative importance of each of the hormones being assessed by regression analysis.

Does Circulatory AMH Regulate the Cardiovascular System?

The known genetic determinants of inherited cardiovascular conditions have a concentration in the TGF β superfamily, particularly the SMAD1/5/8 component, which the BMPs signal through [70]. AMH is BMP-like in its signalling (above) and AMHR2 is present in the developing aorta of mice. We have therefore been interested to determine whether AMH associates with cardiovascular parameters in men.

In healthy men, the level of serum AMH inversely correlates with the diameter of the aorta, with the strength of the correlation being similar to that of the body surface area (BSA), the largest single determinant of aortic diameter [71]. The association between AMH and aortic diameter is independent of all of the major known determinants of the physical state of

the diameter [71]. In men with known vascular disease, higher serum AMH associates with varicose vein disease, and the absence of abdominal aortic aneurysm [71]. Furthermore, the level of AMH in female rhesus monkeys at the time of oophorectomy correlates with smaller plaque size in a model of atherosclerosis [72]. These multiple associations between AMH and the vasculature are intriguing, but we emphasise that there is current no proof that manipulating AMH levels has a direct effect on either the physiology or the cellular anatomy of the cardiovascular system.

BMI and Metabolic Syndrome

Serum AMH levels in men typically do not associate with the person's BMI or other physical traits [71, 73, 74]. Weak negative associations between AMH and BMI have, however, been reported [75] and AMH levels increase when morbidly obese men undergo weight loss [74]. AMH has also been reported to be slightly lower in men with metabolic syndrome [76], although this study is potentially confounded as the men with metabolic syndrome were older than the control group. The association between AMH and metabolic parameters has been more extensively studied in women, with AMH negatively associating with BMI, depending on reproductive parameters [77-80]. These correlations in women are putatively indirect, as AMH levels in intervention studies relate to changes in ovarian function rather than to weight loss or to markers of insulin resistance [81, 82].

This points to the need for further studies to determine if low AMH levels in elderly men is indicative of any systemic disease process or whether they solely reflect changes which are specific to the testes.

CONCLUSION

Most cell types express TGF β superfamily receptors and most cellular environments contain multiple TGF β superfamily ligands, from multiple sources. Circulating AMH may add a gonadal influence to this complex signalling mechanism, albeitly an influence that may be modulated by active regulation of the cleavage of AMH. The physiological significance of circulatory AMH is largely unknown, and only beginning to be investigated. The cardiovascular system is a potential target for circulating AMH, although much further work is needed to prove this. In the age of mass screening, the functions of circulating AMH may be slowly discovered from meta-analyses of large datasets, rather than by hypothesis-driven discovery. The understanding of the basic endocrinology of AMH may facilitate its use as a biomarker. However, many hormones have therapeutic uses, and one of the more compelling reasons for AMH to be studied as a hormone is the prospect that the manipulation of circulating AMH levels or its cleavage may one day have clinical utility.

Questions for Further Research

1. Is AMH signalling restricted to reproductive organs or does circulating AMH affect multiple organs?
2. Is proAMH cleaved to AMH_{N,C} after secretion from the gonads, either in the circulation or in tissues that express both AMH receptors and cleavage enzymes?
3. Does the age-related decline in the testes involve concurrent loss of all testicular hormones? If not, are there sub-types of age-related hypogonadism with different hormonal profiles.

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