Circulating anti-Müllerian hormone in the older man

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Abstract

This thesis provides the first description of circulating anti-Müllerian hormone (AMH) in older men. AMH is a gonadal hormone, with overt function in the generation of the male phenotype during development, and a biomarker for ovarian reserve in women. AMH is one of four circulating hormones produced by the adult testes, and assumed to have no functional significance in men. Therefore there is an absence of information regarding basic physiology of AMH in the blood of men. This thesis posits that AMH may have cryptic functions, with putative interactions to the other testicular hormones. AMH also belong to the TGFβ superfamily of cytokines with ubiquitous roles and convergent intracellular signalling in a contexual manner. The role of AMH may become more evident in a model of dysregulated homeostasis, such as that seen with ageing and frailty.

This PhD examined a local cohort of 223 men from 19 years to 90 years old for their circulating AMH levels. Three testicular hormones were concurrently examined for interactions with AMH; Inhibin B (InhB), testosterone, and insulin-like peptide 3 (INSL3). The serum profiles of these four hormones were examined throughout the day, and across the age spectrum with a focus on men aged 70 years and older. A small group of older women were also examined for their circulating AMH level to determine for sexual dimorphism with ageing. The extent of cleavage of AMH precursor protein was also studied, and compared between men and boys. A pilot study was concurrently set up to test for the feasibility of correlating AMH and other hormones to functional traits associated with ageing.

In healthy young men, circulating AMH and INSL3 levels were both stable throughout the day and independent to each other. This contrasted to the overt diurnal pattern exhibited by testosterone and InhB.

Circulating AMH level varies significantly between men, with age-related decline. AMH level was partially correlated to the other Sertoli hormone InhB which became divergent in older men. These findings were concordant with examination of a distinct cohort of older men in the North Island of New Zealand. Both cohorts however had average circulating AMH levels which were lower than a third age-matched cohort of age with healthy cardiovascular status. Circulating AMH was largely sexually dimorphic with ageing, but not invariantly. A minority of older men lacked AMH like all older women.

All four circulating testicular hormones are independent in younger men. All four testicular hormones exhibits age-related decline, resulting in multiple combinations of testicular hormonal deficiencies. A minority of older men was deficient in all testicular hormones, similar to all postmenopausal women.

The precursor hormone of AMH in blood was strongly linked to its total AMH level. However the extent of its cleavage was distinct to the circulating AMH concentration, and also to the other testicular hormones. AMH was cleaved more in both younger and older men, when compared to boys, with no overlap in their ranges.

Amongst men over 70 years, circulating AMH showed a relationship to cognitive function that was highly statistically significant. This was independent to the other testicular hormones. The extent of its cleavage correlated to physical function and weight.

AMH is present in the circulation in men, including the oldest old. AMH may play a role as a chronic reporter about the state of the testes that is independent to the other three testicular hormones. This has relevance to the understanding of hypogonadism in the older male.

Publications

Papers published from the thesis to date:

1. **Chong, Y.H.**, Campbell, A.J., Farrand, S. and McLennan, I.S. (2012) Anti-Müllerian Hormone level in older women - detection of granulosa cell tumor recurrence. International Journal of Gynecological Cancer 22: 1497-1499.

2. **Chong, Y.H.**, Dennis, N.A., Connolly, M.J., Teh, R., Jones, G.T., van Rij, A.M., Farrand, S., Campbell, A.J. and McLennan, I.S. (2013) Elderly men have low levels of anti-Müllerian hormone and inhibin B, but with high interpersonal variation: a cross-sectional study of the Sertoli cell hormones in 615 community-dwelling men. PLoS One 8: e70967

3. **Chong, Y.H.,** Pankhurst, M.P. and McLennan, I.S. (2015) The daily profiles of circulating AMH and INSL3 in men are distinct from the other testicular hormones, inhibin B and testosterone. PloS One e0133637.

4. Pankhurst, M.P., **Chong, Y.H.** and McLennan, I.S. (2014) ELISA measurements of anti-Müllerian hormone (AMH) in human blood are a composite of the uncleaved and bioactive cleaved forms of AMH. Fertility and Sterility 101:846-850.

5. McLennan, I.S., **Chong, Y.H.**, Kawagishi, Y., Pankhurst, M.W. (2015) A critical evaluation of whether circulating anti-Müllerian hormone is a hormone in adults, with special reference to its putative roles in men. In D. Seifer and R. Tal (Editors), Anti-Müllerian Hormone: Biology, Role in Ovarian Function, and Clinical Significance, in press Nova Science.

6. **Chong, Y.H**., Pankhurst, M.P., and McLennan, I.S. (2016) The testicular hormones AMH, InhB, INSL3 and testosterone can be independently deficient in older men. J Gerontol A Biol Sci Med Sci (in press).

7. Pankhurst MW, **Chong Y.H.**, McLennan IS. (2016) Relative levels of the proprotein and cleavage-activated form of circulating human anti-Müllerian hormone are sexually dimorphic and variable during the life cycle. Physiol Rep 4(9):pii:e12783.

The published papers were written as stand alone documents. This thesis has been drafted as a coherent document, with logic that flows from Chapter to Chapter. Consequently, the introductions and discussions of the thesis and the associated papers have arguments in common, but the phrasing and the order of the arguments vary substantially between the documents. The data in publications 1-3, and 6-7 correspond to Chapters 3-7 of the thesis. The other authors either provided documented serum and/or provided intellectual assistance (AJC, SF, MWP, ISM). I provided partial first drafts of these papers, with the final versions written by ISM.

I contributed documented serum samples, intellectual input and some technical assistance to publication 4., the manuscript that was primarily drafted by the other authors. This paper provides the foundation for Chapter 6 of the thesis.

Publication 5 is an invited review written by ISM, with intellectual input from the other authors. It refers extensively to the data in this thesis.

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List of Abbreviations

3MS	Modified mini mental state examination
6MWT	Six minute walk test
ACVR2A	Activin receptor type-2 A
ACVR2B	Activin receptor type-2 B
AMH/MIS	Anti-Müllerian hormone (also known as Müllerian Inhibitory substance
$AMH_{N,C}$	Cleaved form of anti-Müllerian hormone
AMHR2	Anti-Müllerian hormone receptor type-2
ANOVA	Analysis of variance
API	Anti-Müllerian hormone prohormone index
BMI	Body Mass Index
BMP	Bone morphogenetic protein
BMPR2	Bone morphogenetic protein receptor type-2
ELISA	Enzyme linked immunosorbent assay
Fig.	Figure
FSH	Follicle stimulating hormone
GDF	Growth differentiation factor
GDS	Geriatric depression scale
HCG	human chorionic gonadotropin
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
InhB	Inhibin B
INSL3	Insulin-like peptide 3
LH	Leutinizing hormone
LiLACS	Life and living in advanced age: A cohort study
MMSE	Mini mental state examination
NZ	New Zealand
PCSK	Proprotein convertase subtilisin kexin

preproAMH	Pre-pro-anti-Müllerian hormone
proAMH	Pro-anti-Müllerian hormone
proANP	Pro-atrial natriuretic peptide
proBDNF	Pro-brain-derived neurotrophic factor
proBNP	Pro-brain natriuretic peptide
proNGF	Pro-neurotrophic factor
ref	Reference
SD	Standard deviation
SPPB	Short physical performance battery test
TGFβ	Transforming growth factor β
TGFbR2	Type-2 receptor for transforming growth factor b
TMT A	Trail making test A
TMT B	Trail making test B
TUG	Time-Up and Go test

Chapter 1:

Introduction

1.1 Introduction

Anti-Müllerian hormone (AMH) is a testicular hormone of importance in the generation of the male phenotype during development. AMH continues to be secreted by the testes throughout the life cycle; with many assuming that AMH has no overt function in the adult male. AMH therefore has been poorly studied, resulting in an absence of basic information pertaining to its physiology. This thesis challenges the premise that AMH lacks function in man, and builds a case for its putative role as a signalling testicular hormone in man. This thesis therefore will commence with a description of the endocrine testicular function in man, and review where AMH fits within this context. If AMH does have a function, its mechanistic signalling pathway may be determined by considering AMH as a member of the transforming growth factor beta (TGFβ) superfamily of cytokines. This will be reviewed in the second part of this Chapter.

1.2 The testes contain multiple cell types

The testis is a complex organ comprising of multiple cell types, in different compartments [Fig. 1.1]. Each cell type serves different functions, and interacts with each other in a complex fashion. The simplified understanding of each cell type is as follows. The germ cells undergo spermatogenesis, and supported by the Sertoli cells lining the seminiferous tubules. The Leydig cells within the interstitial compartment generate hormones that develop and maintain the male phenotype.

Other intra-testicular cells, such as the peritubular myoid cells, undergo reciprocal interactions with Sertoli and Leydig cells, both in development and in the adult (1a-d). The interactions between these cell types appear to create linkage and coordination between Sertoli and Leydig cell function. The evidence for this comes from the study of sperm production and/or the development of the testes. To date, there is no proof of linkage between the endocrine functions of the adult testes. Chapter 5 provides the first test of whether such linkages exist.

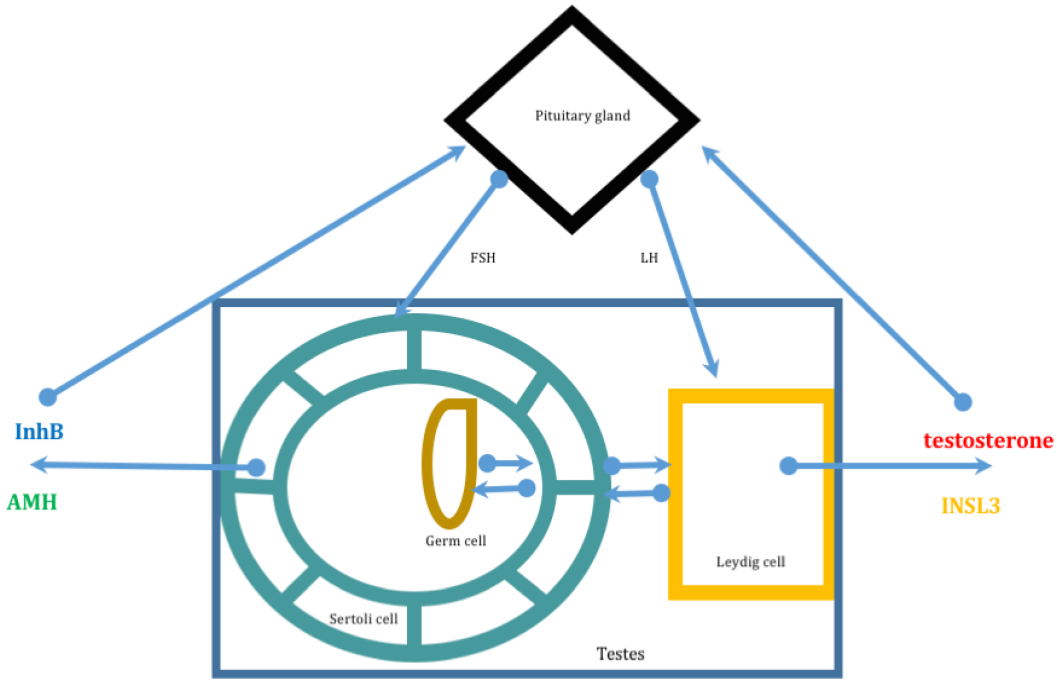

Figure 1.1. Schematic representation of the different testicular endocrine cells, and their relationship to germ cells and the pituitary gland.

1.3 Function of the testes

1.3.1 Generation of the male phenotype in utero

During development, the testes main function is to generate the male phenotype, which involves both virilisation and defeminisation. This occurs at specific stages. A characteristic feature of this developmental stage is the overt phenotype that is generated, in a dichotomy fashion depending upon the presence, or absence of testicular hormones. AMH induces the regression of the paramesonephric ducts (Müllerian ducts). Loss of function mutation in the genes of either AMH or its obligatory type-2 receptor AMHR2 leads to the persistence of the paramesonephric ducts (1). This process occurs independent of other testicular hormones. Testosterone independently induces the development of the mesonephric ducts into the male genitalia. Its absence or loss of function of the androgen receptor results in the lack of virilisation of the genital (2, 3).

The testicular hormones AMH, testosterone and insulin-like peptide 3 (INSL3) also work in a concerted manner to facilitate the descent of the testes from abdomen into the scrotum. The presence of each hormone during different stages in the testicular descent is important, as the absence results in retention of the testes within the abdomen (4-6).

1.3.2 Generation of the adult male phenotype

The final adult male phenotype is generated from puberty, and maintained during adulthood by testicular hormones. Aspects of this phenotype maybe overt and result in sexual dimorphism. Clear examples include the deepening of the voice, development of the penis, androgenic hair growth, changes to anthropometric features such as increased muscle mass and the robustness of bones. There is strong evidence that testosterone is the main mediator of these overt functions, either directly via its steroid receptors (7), or indirectly in conjunction with other hormones, such as growth hormone for height (8).

Whilst adult human exhibit strong sexually dimorphic traits between genders, various grades of phenotype exists within the same gender. Height and muscle strength varies between men, with the variability not strongly explained by differences in their testosterone level (9). The influence of other testicular hormones on the variability is currently unexplored, and they may have modulating roles in the maintenance of these traits. For instance, testosterone is attributed to be important for maintenance of bone mass, and testosterone deficiency results in osteoporosis for some, but not all men. Two other testicular hormones, INSL3 and inhibin B (InhB) are bone regulators, and the disruption in their signalling leads to osteoporosis (10-12). AMH therefore may have a modulating role in the generation of male phenotype that explains the variability between men, but not overtly seen.

Two other testicular hormones, INSL3 and inhibin B (InhB) are bone regulators at an endocrine level. Adult male studies in men and mice have shown that mutation in the sole INSL3 receptor RXFP2 leads to osteoporosis, backed by mechanistic studies (12a). Similarly, serum inhibin level is better at predicting osteoporosis than oestrogen in women, and supported by mechanistic studies showing regulation of inhibins upon bone turnover (12b-d). Since both circulating INSL3 and InhB are solely derived from the testes (30, 165), the distal effect on bone regulation strongly suggest their endocrine function in adult.

1.3.3 Spermatogenesis requires complex interaction between testicular hormones

The study of spermatogenesis in relation to testicular hormones is outside of the scope of this thesis. However a targeted review of testicular hormonal control of spermatogenesis can illustrate the complexity of hormonal and cellular interaction with the testes.

Germ cells are isolated within the seminiferous tubules by the formation of tight junctions (13). This isolation is crucial for spermatogenesis (14, 15). Germ cells are therefore dependent upon the Sertoli cells for support (16). The state of the

Sertoli cell coincides with spermatogenesis, as the number of Sertoli cells partially correlates with the number of germ cells (17). It has been proposed that Sertoli hormones AMH and InhB may serve as surrogate markers for Sertoli numbers (18-20). If so, then either levels of AMH, or InhB, or both may reflect Sertoli cell number. If both AMH and InhB are surrogate markers of Sertoli cell numbers, they are expected to be highly linked, and therefore exhibit strong correlation to each other in blood.

The body is able to influence the process of spermatogenesis by follicle stimulating hormone (FSH) and testosterone, via their Sertoli cell receptors (21). However the interaction is redundant, as the loss of either testosterone or FSH only leads to partial reduction in germ cells, with the complete disruption of spermatogenesis only evident with abolishment of both hormonal receptors (22). Sertoli cell is able to modify this interaction and provide feedback. InhB negatively regulates FSH (23, 24), and Sertoli cell also secretes androgen-binding protein that sequesters testosterone within the testes (25, 26). This effect of Sertoli protein production can be mediated directly by different state of spermatogenesis (27). Hence the state of the Sertoli cell plays a pivotal role in the cross-talk between the body and spermatogenesis, and the level of AMH may provide an indication about the state of the gametes.

1.4 Regulation of testicular endocrine cells

1.4.1 Luteinizing hormone regulation of Leydig cells

The two endocrine cell types play major roles in the function of the testes. An understanding of its regulation may shed light as to putative function for AMH. Similar to the regulation of spermatogenesis, the regulation of both Leydig cells and Sertoli cells are complex. Leydig cells produce two hormones in man; testosterone and INSL3 (28-30). Both are controlled by the pituitary hormone luteinizing hormone (LH) (29-32), and reflects different effects of LH on the Leydig cells. Both intrastesticular and systemic testosterone level fluctuates episodically in reponse to the pulsatile pattern of LH. INSL3 is invariant to acute changes of LH, but chronic depletion of LH leads to a drop in INSL3 (29-32). The

two Leydig hormones therefore report different states of the Leydig cell; testosterone on the acute regulation of LH, whilst level of INSL3 reflect the basal state of Leydig cells (33).

1.4.2 Sertoli regulation of Leydig cells

There are cross-talk between the Leydig cells and Sertoli cells. Experimental ablation of Sertoli cells in mice leads to a significant decrease in Leydig cell number, suggesting that Sertoli cell maintains the Leydig cell (34, 35). The influence of Sertoli cells upon Leydig cells maybe due to Sertoli hormonal function. InhB may indirectly affect Leydig cell growth via FSH (36) or directly via its receptor on Leydig cells (37).

AMH exerts several effects on Leydig cell development and function. It is observed that mutant mice with loss of function AMH (38) or its receptor (39) exhibit hyperplasia of Leydig cells but normal levels of testosterone (40). Conversely, transgenic overexpression of AMH in mice leads to reduction of Leydig cell numbers and testosterone level (40). However, interpretation of this study is limited by the supraphysiological over-expression of AMH at twice the peak concentration of an infant male (41, 42). The effect of AMH on Leydig cell is said to be mediated via its receptor AMHR2 expressed on Leydig cells (42-45). Again, interpretation of these studies are limited as data from in vivo studies is lacking. The effect of AMH upon Leydig cell development or its function therefore is unclear based on current literature. Putatively, AMH may exert a permissive role in Leydig cell development (42) much like what has been demonstrated with testicular hormones during development, and effect of LH on INSL3 [Chapter 1.4.1]. There is also a need to determine normal levels of circulating AMH in men, to guide physiological replication of AMH levels in animal studies.

1.4.3 Regulation of Sertoli cells

The regulation of Sertoli cell is equally as complex as Leydig cell, and is lifestage dependent, involving FSH, testosterone, thyroid hormones, activins, and other paracrine factors (44). It is known that Sertoli cells undergo a period of

proliferation during the pubertal transition, then terminally differentiate with stable numbers in adulthood (45). Since Sertoli cells express androgen receptors during puberty, it has been theorised that testosterone down-regulate Sertoli cells production of AMH during puberty, with this effect persisting into adulthood (46). Consequently, circulating AMH level is thought to be suppressed by peak adult testosterone level (47, 48) and therefore not thought to have any functional significance in adulthood.

Testosterone appears to work in concert with FSH to regulate Sertoli cells, similar to their effect on spermatogenesis. Mice that lacked FSH or FSH receptors have reduced number of Sertoli cells during adulthood, even in the presence of testosterone replacement (49). In a separate study, mice that lacked androgen receptors specific to the Sertoli cells only have preserved number of Sertoli cells (50), indicating that androgen effect is indirect. Additionally, mice that lacked both FSH receptor and androgen receptor in their Sertoli cells have diminished number of Sertoli cells (51). FSH effect on Sertoli cell appeared to stage specific, as Sertoli cells become resistant to the effect of FSH following puberty (52). If testosterone regulates the production of Sertoli cell hormones, then the levels of testosterone and InhB and AMH should link during adulthood, or when testosterone level changes. The first tests of whether AMH and testosterone levels associate are described in this thesis [Chapters 3, 6 and 7].

There is an absence of evidence of direct androgen regulation of Sertoli cells in adult (48). This contrasts with the large and complex body of literature that examines the interplay between Leydig cells and Sertoli cells during development and puberty (21). Further in-depth review of the interaction between the two cell types have not been undertaken, as this does not translate to adulthood or the ageing endocrine testes, which forms the focus of this thesis.

1.5 Circulating levels of testicular hormones

1.5.1 Levels of testosterone, INSL3 and InhB

The circulating levels of testicular hormones reflect their endocrine functions, particularly of testosterone. In the first three months of life, testosterone, INSL3 and InhB all experience a surge in their circulating levels due to the elevation of gonadotropins (53, 54). This quickly subsides with the Leydig hormones during childhood becoming queiscent (55). In contrast, InhB level is detected in blood of boys, at approximately halved that of peak adult level (56-58). In puberty, all three hormones increase in their levels to reach their peak adult levels (56, 58). The prevailing theory is that boys' testes are non-functional in childhood, due to the their low levels of testicular hormones and a lack of overt sexual dimorphism between boys and girls beyond what is established in utero, and not to the same extent as in adulthoood.

1.5.2 Circulating levels of AMH in males and females

The level of circulating AMH in males are exclusively derived from the testes (41, 59-61). At the commencement of this PhD levels of AMH is unknown beyond puberty. In contrast to the other three hormones, circulating AMH level is thought to peak in boys, with stable levels during childhood (41, 62-64). Females also secrete AMH from their ovaries into blood, which exists in very low levels in girls. It has been postulated that this distinction in serum AMH levels between boys and girls may contribute to subtle sex bias (65). Evidence to support this theory comes from the correlation between AMH and autism in boys (66), the restraint on the speed of maturation in boys (62), and the preference of young male mouse pups for novelty behaviour (67). If this is true, then AMH may have similar role in maintaining the male phenotype in adulthood.

Male circulating AMH levels decline during puberty down to an order of magnitude from peak childhood level (41, 68). It is therefore assumed that AMH has no function in men due to the low levels. However, AMH at concentration typical of adult men is able to induce the survival of embryonic motor neurons in

vitro (69), suggesting that it may be possible for AMH in men to elicit intracellular signalling. This will be discussed in greater detail in the TGFβ superfamily section of this Chapter.

Female circulating AMH level peak during young adulthood, and is derived from the granulosa cells surrounding the germ cells (70, 71). Circulating levels of AMH in women decline in parallel with the decline of their ovarian reserve of follicles (72, 73), with an universal cessation of AMH level near menopause (74, 75). Circulating AMH level informs about the state of the germ cells in women, with this being utilised clinically as a biomarker for fertility (76, 77). This sets the possibility that AMH level in men may play similar role as a biomarker for the state of the germ cells.

1.5.3 Implications of circulating testicular hormonal levels to late onset hypogonadism

Numerous population studies have shown that circulating testosterone level decline in men as they age. The proportion of older men having testosterone levels in the hypogonadal range of younger men increased to nearly 50% of men in their 9th decade of life. (77a-c, 172). The low androgen state of these older men do not consistently correlate with classical symptoms of pathological low androgen status commonly seen in younger men (172). This has led to the emergence of late onset hypogonadism as a clinical concept to describe pathological low testosterone level in the older person (77d), with diagnostic criteria being low levels of circulating testosterone level and concurrent specific sexual dysfunction symptoms, resulting in an estimated prevalence of only 2% in older men (77e). The lack of utility of LH in this diagnostic criteria reflects the heterogeneity of LH levels amongst older men with low testosterone levels, with some being elevated, some normal, and others being low (77f-g).

Circulating INSL3 levels in older men have only been profiled in one cross-sectional study, with no correlation to testosterone or to LH observed (134). This

indicates that the two Leydig hormones exhibit distinct profiles in ageing, and are not regulated together at the Leydig cell level or at the pituitary level by LH.

The profile of circulating InhB level in older men is similarly variable to ageing and to the FSH level (176-178), whilst circulating AMH level in older men was unknown at the commencement of this PhD. The description of late onset hypogonadism is therefore incomplete when only analysing either Leydig cell hormones or Sertoli cell hormones, with the study of all four hormones required to better understood the biological changes associated with the ageing endocrine testes.

1.6 AMH as a TGFβ superfamily member

AMH has one overt function in the male, which is to induce the regression of the paramesonephric ducts (Müllerian ducts), by activating the AMH-specific receptor, AMHR2 (1). For this function, AMH behaves like a classical hormone, producing an effect that is overt and independent of the presence or absence of other regulators. This model of AMH action does not assist the search for the functions of AMH in the adult male, as men with a null mutation of AMH do not exhibit any overt symptoms, beyond cryptorchidism due to the retention of the paramesonephric ducts (1, 78).

This thesis proposes that AMH should be considered in the context of being a member of the TGFβ superfamily of cytokines. This family of 35 cytokines, including AMH and InhB, converge onto a limited number of receptors to activate its canonical signalling pathway (79, 80). Despite the limited number of combinations, members of this family is ubiquitous in their function spanning from development, to homeostasis and pathology (81, 82). Diversity and pleiotropism is achieved through complex interactions between the ligands, receptors and associated binding proteins (83). The expression of each ligand varies between different cell types, and between different stages of life from development through to homeostasis, to ageing and pathology. The influence of the ligand can also exist from being fully redundant with other ligands, to being

fully independent. Consequently, null mutations of the TGFβ superfamily members often results in mild deviation from the normal phenotype (84-87), with this being evidence of the independent functions of the ligand. If AMH operates in a similar manner, then it may have broad functions in inducing and maintaining the male phenotype, in concert with bone morphogenetic proteins (BMP) ligands (65). Hence, AMH maybe misconstrued as not having any functional significance in adults, if it is exclusively seen as signalling independent to the other ligands.

1.6.1 Cleavage of the prohormone

Protein hormones and cytokines are synthesised as precursor proteins that undergo processing prior to binding to its target receptors. The classic model is an initial production of protein called preproprotein, which is thought to undergo cleavage of its signal peptide intracellularly to generate a proprotein. This proprotein is then cleaved, to yield its N-terminal and C-terminal components, with the cleavage occurring either intracellularly during protein synthesis or at multiple extracellular sites. In the case of the TGFβ superfamily members, it is the C-terminal component that binds to the target receptors. The N-terminal also has diverse functions, and therefore remain non-covalently associated with the C-terminal for some TGFβ superfamily members. This includes the susceptibility of the proform to cleavage (88), and after cleavage the regulation of the stability (88) and transport and the complex (80). AMH is typical of this scenario, where the proAMH is cleaved to its C-terminal receptor binding form with the N-terminal non-covalently bonded as $AMH_{N,C}$ (89). Consistent with known N-terminal function, free C-terminal AMH do not exist in biological fluid including blood (90), and it is thought that the $AMH_{N,C}$ dissociate with influence by the target receptor (91).

There is an emerging awareness of the possible significance of pre-protein in biology, beyond its role intracellularly. The signal peptide cleaved from some preproproteins can be detected in the circulation (92, 93), with some correlation to pathology (94). The most topical in the New Zealand setting are studies of the signal peptides from the natriuretic peptides, which are cytokines central to the

pathophysiology of cardiovascular diseases (95, 96). The natriuretic signal peptides have been shown to be correlative to certain cardiovascular traits, and research is ongoing into its potential role as a cardiovascular biomarker (92, 94, 97). It is currently unknown if preproAMH exists in circulation.

Similarly, the proprotein were initially thought to have no functional purpose beyond being a substrate for the generation of the C-terminal. However, this may not be universally correct, with the strongest evidence for function of proprotein coming from the study of proneutrophins (proNGF and proBDNF). The proneutrophins are able to activate receptors independent from their C-terminal site (98, 99). This generates a mechanism where the ability of the target cell to cleave the proneurotrophin dictates the final phenotype.

In other instances, the role of the intact proprotein prior to cleavage is unknown but thought to have biological significance. Some proproteins are concurrently detected in circulation as well as the cleaved active peptide. Consequently, there is emerging interest in the exploration of the extent of the cleavage of the proprotein as potential biomarkers. For instance, both proinsulin and the activated insulin are detected in blood of people with normal glucose metabolism and those with diabetes mellitus (100, 101). The relative ratio of proinsulin to insulin in blood have been shown be different with ageing and with a glucose challenge (100). Subsequently the ratio of the two hormones have been suggested as potential biomarker for prediction of development of diabetes mellitus due to pancreatic beta cell dysfunction (100, 102).

Differential cleavage of the proprotein may yield different active cytokines that have distinct functions, in a context specific manner. For example, pro-opiomelanocortin is a prohormone synthesised in the anterior pituitary gland, and cleaved by several prohormone convertase enzymes. The cleavage is tissue specific, and yields several distinct cytokines and hormones, including adrenocorticotropic hormone in the pituitary gland, and melanin in the skin (103). The prohormone chromogranin A is another example. Chromogranin A is a neuroendocrine prohormone that yields multiple active peptides with distinct

functions (104). Chromogranin A itself is found in blood and is utilised as a biomarker for diagnosis of neuroendocrine tumours such as carcinoid tumours and pheochromocytoma (105, 106). The regulation of the cleavage activity of a propeptide therefore may influence different bodily function to the active peptide itself.

There is scarce knowledge about the processing of precursors of AMH, and an absent of information pertaining to its cleavage in men. Intact proAMH is known to exist, with its presence in blood only being documented since the commencement of this PhD (90). ProAMH is putatively cleaved by several prohormone convertase enzymes that has wide ranging functions, such as furin [Chapter 6.1]. The role of proAMH has not been elucidated. Given the possibility of functional significance of prohormones, as well as the cleavage extent being a biomarker for other prohormones, this provides the basis to studying proAMH and the extent of its cleavage in circulation to the cleaved form $AMH_{N,C}$. The regulation and influence of proAMH and its cleavage extent may be distinct to the ability of AMH to interact with its receptor and downstream intracellular signalling.

1.6.2 Canonical receptor activation

The canonical receptor activation for all TGFβ superfamily members involves shared signalling components: 35 ligands converge onto 5 type-2 receptors which associates and activate 7 type-1 receptors [Fig. 1.2] (79, 107). Each receptor type only interacts with certain members of this superfamily. Consequently the specificity of the ligands to the different type-2 receptors is used to subdivide this superfamily into the following groups: the TGFβ (TGFβR2), the activins (ACVR2A), the BMP (BMPR2, ACVR2B) and AMH (AMHR2). The largest subgroup is the BMP, which also includes the growth and differentiating factors. Each of the type-2 receptors can associate with a subset of the type-1 receptors, resulting in certain type-1 receptors being able to bind to multiple type-2 receptors [Fig. 1.2]. The type-1 receptor defines downstream intracellular signalling by phosphorylating specific intracellular proteins called receptor-SMADs, which can be broadly divided into two groups: those that are activated by the BMPs and a minority of TGFβ subfamily signalling (SMAD1/5/8), and members that belong to the TGFβ/activin/inhibin group (SMAD2/3). The canonical receptor signalling therefore results in 35 ligands interacting with distinct type-2 receptors which converges to 2 groups at an intracellular level, due to shared type-1 receptors. This may be central to the understanding of AMH signalling, as the type-1 receptors and receptor-SMADs activated by AMH are common to the BMP group (79, 108). AMH therefore may signal as a BMP ligand, with phenotypic features common to the BMPs (65).

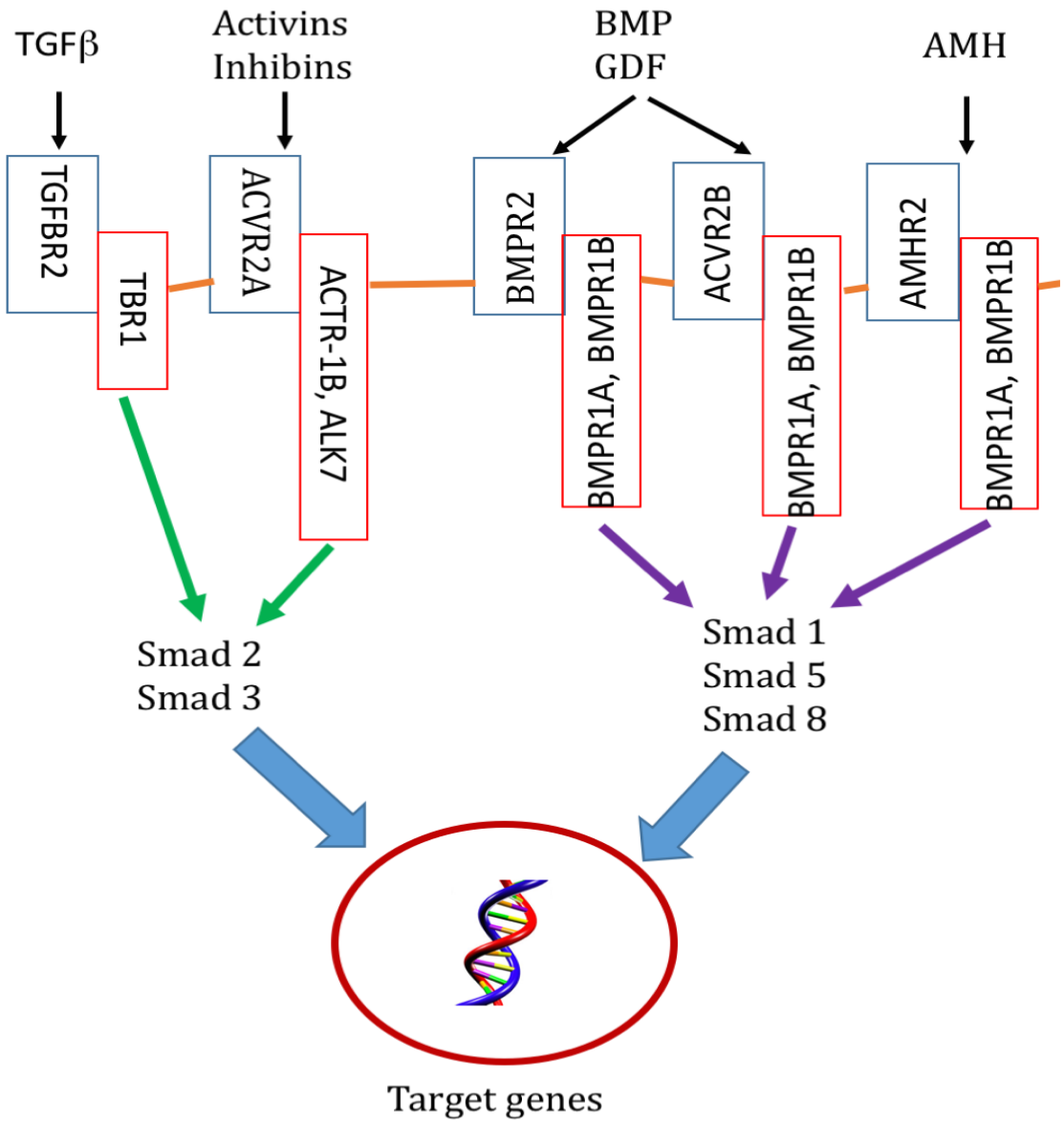

Figure 1.2. Canonical signalling pathway of TGFβ superfamily ligands and their receptors.

The type-1 receptor (red box) used by AMH signalling is shared by the BMP and GDF ligands, despite their differences in type-2 receptors (blue box). AMH, BMPs and GDFs all activate the common receptor SMADs 1/5/8. Figures are adapted from (65, 79).

InhB acts as an antagonist to the activins, by competitively binding to the same type-2 receptors as activins, but not recruiting the type-1 receptors and hence not inducing any intracellular signalling of the activin group (109, 110). Both InhB and AMH are products of the Sertoli cells, but their effect on the intracellular pathways are divergent. This suggests that the Sertoli cell hormones may have independent functions.

AMH has been thought to lack function in men, as the circulating level of AMH in the post-pubertal male is much lower than that of boys [Chapter 1.5.2]. When viewed in the context as a member of the TGFβ superfamily, there maybe a possibility that AMH may have function at adult concentration. A defining feature of this superfamily is the ability to induce cell signalling by ligands across a wide range of concentrations by orders of magnitude (111). This is achieved with the use of binding proteins that potentiate the binding of the ligand to the receptor. For instance, betaglycans acts as a co-receptor for InhB on ACVR2 receptor in certain tissues to result in antagonism of activin, whereas the absence of betaglycans result in a lack of binding by InhB (112). Again, this illustrates the contextual nature of signalling in this superfamily. The existence of a binding protein for AMH is currently unknown,. The activin binding protein Follistatin may be a putative candidate, and forms the basis of a colleague's PhD thesis submission.

Variations in AMHR2, the specific type-2 receptor for AMH is known (113), and influence the ability for AMH to signal (114). Consequently, there is increasing interest in the exploration of the AMHR2 polymorphism and gonadal function in the female (115-119).

The signalling pathway for AMH is known for induction of paramesonephric duct regression. This involves the binding of AMH onto AMHR2, and recruiting two of the BMP type-1 receptors, resulting in the activation of intracellular signalling common to the BMP subgroup (120, 121). The type-1 receptors have been shown to be redundant in the generation of the overt phenotype persistent Müllerian duct syndrome (122, 123). It is currently unknown if AMH can bind to

other type-2 receptors beyond AMHR2, but the possibilities have been set up in the above sections. AMHR2 is expressed by tissues beyond the testes (124, 125), particularly in the nervous system (69, 126, 127). This sets up a direction to explore for putative association of AMH in the central nervous system. Alternatively or concurrently, AMH may function like a BMP ligand. As stated above, its function may not be obvious, unless there is significant disruption to other ligands that share similar downstream signalling. A potential model to examine for putative AMH function may be found in the older person with naturally occurring states of impaired homeostasis, particularly when associated with frailty [Chapter 7].

1.7 Objectives of the thesis

This thesis aims to examine a circulating hormone in men, for which there is no known function. The receptor for AMH is present in multiple tissues, but this does not prove its function [Section 1.6.2]. AMH is found in circulation in young men and women, but scarce information is known regarding its physiology following puberty [Section 1.5.2]. Null mutants for AMH are extremely rare in humans, which means that the study for putative AMH action has to begin with association studies, to correlate phenotypic traits, including diseases, and circulating AMH levels. All postmenopausal women lack AMH, whilst the profile of circulating AMH in older men is unknown [Section 1.5.2, Chapter 4.1]. If AMH exhibits sexual dimorphism with ageing, then the lack of AMH in older women may hold clues as to putative functions of AMH. Ageing changes such as cardiovascular disease profiles and osteoporosis differ between men and women, with these changes historically attributable to differences in sex steroid levels (127a-b).

Evidence from mice have shown that AMH is regulator of neurons, which is not easily studied in younger people due to multiple redundant biological systems [Section 1.6.2]. If AMH has a functional significance, perturbations in its levels would be more likely to be observed in older men compared to younger men, as older people exhibit more dysregulation in their homeostasis with ageing. This is profiled in the context of AMH as a Sertoli hormone [Chapter 4] and also in the context of AMH as a testicular hormone in relation to the Leydig hormones [Chapter 5]. The profiling of proAMH and the extent of its cleavage in men is described in Chapter 6. There is no *a priori* hypothesis as to the function of AMH in men. However, if AMH does have broad function, then its perturbation is more likely to be observed in men that lacks physiological reserve to maintain homeostasis. This is more frequently seen in the older person, particularly those with frailty. Chapter 7 will describe the first putative association study of circulating AMH, in the context of its cleavage and other testicular hormones, in a cohort of older men. The study incorporates my area of interest as a practicing

clinician in both endocrinology and geriatric medicine, and consolidates both clinical acumen and laboratory skills learnt during the PhD study.
Before any correlative studies can be performed, the stability of circulating AMH throughout the day has to be determined, and whether it is subjected to any sporadic influence or exhibit moment-moment relationship with the other testicular hormones [Chapter 3].

This thesis is an exploration into an uncharted territory in biological and medical science. This requires observational studies, which forms the basis of this PhD thesis. Where levels of circulating AMH is varying, this may hold clues as to its function, and enables the generation of specific hypotheses for future studies.

Chapter 2:
General Methods

Common methodological techniques employed throughout the thesis are described in this Chapter. Specific methodologies are discussed in individual Chapters that are pertinent to that Chapter.

2.1 Serum Hormonal Analyses

2.1.1 Processing of Sera

Venous bloods were collected into 6 mL BD Vacutainer red top test tubes from the antecubital vein by venepuncture using a 20G hypodermic needle encased in a plastic container. Blood samples were left to clot at room temperature in upright position for 60 minutes. Sera were obtained by centrifugation at 2,000 g for five minutes, immediately snapped frozen with liquid nitrogen and stored at minus 80^0C freezer in multiple aliquots. Samples were thawed only once in 4^0C fridge for at least an hour prior to enzyme linked immunosorbent assay (ELISA) assay.

2.1.2 ELISA description

Circulating hormones were mostly assayed by ELISA, either of the sandwich or competitive type [Table 2.1]. ELISA assays allows relative quantification of specific proteins within a sample, in relation to a set of protein standards with known concentrations values. The general techniques are described below. To reduce interassay variability, all blood samples were assayed in a single batch for each hormone.

Table 2.1 Commercial ELISA kits employed in PhD

Hormone	Company	Name (Catalogue)	Sensitivity	Intra and Inter assay values
AMH	Beckman Coulter	Gen II ELISA (A73818)	1.1 pM	5.4% / 5.6%
InhB	Beckman Coulter	Gen II ELISA (A81303)	1 pg/mL	5.6% / 6.2%
INSL3	Phoenix Pharmaceuticals	INSL3/RFL (human) fluorescent immunoassay kit (FEK-035-27)	19.6 pg/mL	10% / 15%
Testosterone	Endolab Christchurch	Testosterone ELISA	0.3 nM	7.6% /12.5%
LH	Alpco	Leutinizing hormone ELISA (11-LUTHU-E01)	0.2 IU/L	4.5% / 5.1%
FSH	Alpco	Follicle stimulating hormone ELISA (11-FSHHU-E01)	1 IU/L	5.8% / 7.7%
Cortisol	R&D	Cortisol assay (KGE008)	0.071 ng/mL (0.20 nmol/L)	9.2% /21.2%

The primary antibody to a particular epitope of the protein is either added to the ELISA wells (testosterone assay) or is already pre-coated by the manufacturer. Sera and standard solutions in measured amounts are incubated in the individual ELISA wells for set timed intervals at room temperature, which facilitates binding of the antigen to the primary antibody. In the context of a competitive ELISA such as cortisol assay, a fixed amount of horseradish peroxidase (HRP) labelled cortisol is concomitantly added to the wells to compete with the endogenous cortisol for the primary antibody. The greater the amount of endogenous cortisol, the fewer binding sites are available for the HRP labelled cortisol. The wells are subsequently washed with a biological detergent, leaving only the primary antibody-antigen attached to the wall of the ELISA wells. In the case of a sandwich ELISA, a second antibody labelled with biotin is then added, specific to another epitopic site of the protein, thereby "sandwiching" the protein of interest between two antibodies. HRP is consequently added that attach to the biotin labelled detection antibody. In both ELISA types, a substrate, usually tetramethylbenzadine is added and incubated for a fixed time period, allowing the HRP to catalyse a colourimetric reaction, which is stopped by the addition of sulphuric acid. The degree of HRP mediated enzymatic reaction is quantified by measuring the light absorbance VICTORTM X3 Multilabel Plate Reader (PerkinElmer) at 450 nm wavelength, corrected for the background 570 nm wavelength. The values are plotted against a standard curve obtained from the standards generated using the best-fit equation (usually quadratic curve, or 5-point parametric curve). In the context of the sandwich ELISA, the value of the light absorbance is proportional to the amount of protein/antigen tested. For the competitive ELISA, there is an inverse relationship between the absorbance and the amount of protein due to the competition with HRP labeled protein. All ELISAs were performed manually, and each sample was assayed in duplicate, with the final results obtained by taking the average of the two samples. The AMH assay CV was tested by my laboratory, with inter and intra-assay reported in Table 2.1.

Testosterone was initially assayed with the R&D Testosterone ELISA kit (KGE010) but high intra and interassay coefficient of variation with duplicate

samples preclude further utility of the data for analyses. The decision was made to engage the clinical laboratory for Christchurch Hospital (Endolab, Christchurch), using a competitive ELISA developed in-house (128).

2.1.3 ProAMH ELISA

The Beckman Coulter AMH Gen II AMH ELISA reports total AMH, which is an aggregate measure of proAMH and $AMH_{N,C}$ found in blood (Chapter 6.1)(90). A laboratory grade ELISA was developed to quantify the amount of proAMH in serum (129), by modifying the procedure utilised in the AMH Gen II assay kit. The capture antibody in the Gen II assay binds to AMH_C, and the detection antibody binds to AMH_N. The ProAMH ELISA dissociates $AMH_{N,C}$ with deoxycholate, which removes AMH_N and prevents its binding to the detection antibody. ProAMH is unaffected by the treatment as the protein is uncleaved, and therefore the AMH_N and AMH_C species do not dissociate. Two 15-minute, 150-µL wash steps of 0.2 %w/v sodium deoxycholate were inserted into the AMH Gen II protocol after the sample-binding step.

Intra and inter-assay variations were reported to be 8% and 13% respectively. The limit of detection for total AMH and proAMH were measured by my laboratory, and were 1.1 and 0.9 pM respectively (129).
The AMH prohormone index (API) was calculated as the relative proportion of proAMH, expressed as a percentage of total AMH (API = [proAMH]/[total AMH] × 100). The level of $AMH_{N,C}$ was estimated as [total AMH] − [proAMH].

2.1.4 INSL3 assay description

The INSL3 enzyme immunoassay (EIA) [Table 2.1] is a fluorescent immunoassay that uses the competitive ELISA principle. The EIA wells are pre-coated with a secondary antibody, which captures the detection antibody. Diluted samples and standards are added to the wells, and compete for primary antibody binding in the presence of biotinylated-INSL3 peptide. Only the biotinylated-peptide will bind the HRP, which in turn catalyses the substrate reaction. The relative immunofluorescence level is quantified by the fluorescence microplate reader with excitation and emission wavelengths of 320 nm and 405 nm respectively set for detection. The relative degree of fluorescence is inversely proportional to the amount of amount of INSL3 in a sample, and solved by standard curves using 5-point parameters equations.

2.1.5 Limitations of ELISAs

2.1.5A. AMH field notice

AMH was measured using Beckman Coulter AMH Gen II ELISA commercial kit. In June 2013, Beckman Coulter issued a field notice alerting to underestimation of AMH due to interference within samples, and advised pre-diluting the samples for one hour at room temperature with assay buffer prior to assay to nullify the effect of the interference. Most of the AMH data within this thesis had to be re-assayed, with the exception of sera from the Vascular and the LiLACS NZ cohorts, due to sample availability.

On average, AMH values post field notice were 1.6 times greater than pre-dilution (standard deviation =0.58, n=316), with this difference being highly significant ($p<0.0001$, two tailed paired Student's *t*-test). The correlation between the old and newer data sets was high (Pearson's linear regression $R^2=0.87$, $p<0.0001$). These findings are consistent with published literature on AMH values in women (130-133), and the first to be described in men. The absolute values of AMH vary between pre- and post-Field notice data, but the relative values are still preserved. Therefore, conclusions drawn from studies utilising the AMH assay pre-Field notice remains valid, with the information still relevant.

2.1.5B Comparisons of results between studies

ELISA kits allow the quantification of proteins by comparing the measured value against a reference curve generated from standards measured within the same kit. Currently there is no agreed international standard for AMH and INSL3, which precludes the ability to compare the absolute hormonal values derived from different studies that utilise different ELISA kits. Additionally, different laboratory environmental conditions can also affect the results of the ELISA output, hence necessitating the practice of local laboratory reference ranges for comparisons.

The distribution of hormonal values however can be meaningfully compared, as the ranked distribution between individuals should remain invariant between different studies. The profiling of circulating male INSL3 is a recent phenomenon, with differences in absolute mean values of INSL3 observed between different cross-sectional studies (30, 134). However, when the individual values are normalised against other studies, the distribution of INSL3 from this PhD study is similar to the other studies (30, 134) [Fig. 2.1]

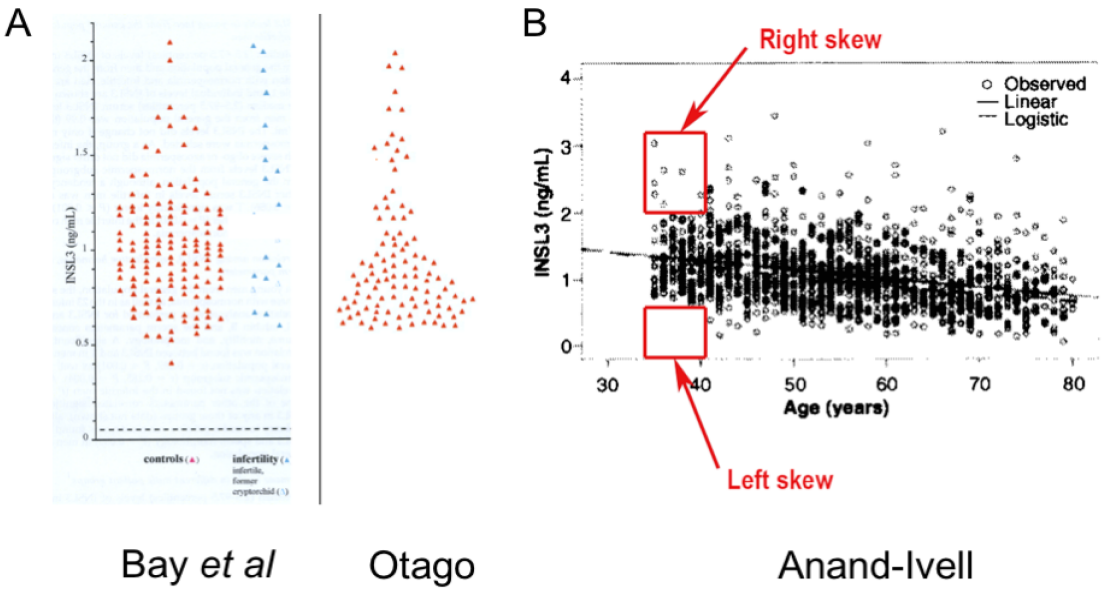

Figure 2.1. Circulating INSL3 levels between the Otago cohort and two other cohorts.

Each symbol represents an individual's INSL3 level. A. The distributions of INSL3 in the study by Bay *et al* (30) is similar to the distribution in the Otago cohort, when the Otago values are normalised to achieve the same mean as that in Bay *et al.*

B. The distribution of INSL3 in the Australian cohort of men was positively skewed (134), with similar proportions of men with high INSL3 values as the Otago cohort.

2.1.5C Comparisons with alternative immunoassays or assay platforms

The perceived gold standard for the assay of testosterone in the laboratory setting has been mass spectrometry, with better sensitivity for measurement of very low levels of testosterone. However, a large observational study comparing measurement of circulating testosterone by both mass spectrometry and immunoassay have found high correlation of results between the two platforms, with no advantage of using mass spectrometry (30a). The utility of mass spectrometry for measurement of testosterone in this PhD study was explored, but not pursued further.

Immunoassays for both AMH and INSL3 are currently still at a research stage, although the Beckman Coulter Gen II AMH ELISA is used clinically within in-vitro fertilisation programmes. This has led to extensive off-label use of this ELISA in clinical research relating to obstetrics and gynaecology. The Beckman Coulter Gen II AMH ELISA was the dominant AMH assay throughout the period of the thesis research. The results from the various competing AMH assays correlate with a high correlation coefficient, indicating that they accurately rank samples by level of AMH. The absolute values from AMH ELISA are less concordant, as there is no international standard for the AMH ELISA. The INSL3 ELISAs are less developed than AMH ELISA, with all INSL3 ELISA being in-house or sold by small companies. The INSL3 ELISAs use competitive binding and are therefore more prone to error than the sandwich technology used in the AMH and InhB ELISAs. As with the AMH ELISA, absolute values can not be compared between studies.

Recently, our research group have identified two species of AMH in blood, proAMH and $AMH_{N,C}$. However, all commercial immunoassays for AMH only report the aggregate or total amount. The significance of this can not currently be assessed, as the bioactivity of circulating proAMH and $AMH_{N,C}$ are unclear. Another factor that may impact on ELISA output is the degree of glycosylation of the protein being measured.

2.2 Calculations and statistical analyses

The levels of circulating hormones and clinical attributes are reported as mean with standard deviation, and/or as median with range. Distributions of the hormonal values were examined for skewness, with statistical significance tested using SKTEST (Skewness and Kurtosis Test for Normality). A minority of men examined in Chapter 4 and 5 had undetectable levels of circulating testicular hormones, whilst some men had low but detectable levels. These men were included in all statistical analyses, thereby avoiding left censoring.

Associations between two variables were tested in univariate analyses using linear regression with Pearson correlation. Multiple variables were examined for their impact on the relationship using partial correlation. Differences between two groups of the same variable were tested using Student's *t*-test, whilst variations between subgroups were examined by one-way ANOVA, and post-hoc analyses with Scheffe's test to investigate for differences between subgroups. P values less than 0.05 are considered statistically significant.

The levels of hormones vary between individuals. If the relationship between hormones (e.g. AMH and InhB) were described as a simple ratio, the resulting distribution would not be symmetrical. Individuals with high AMH would be represented by a range of 1 to infinity, whilst those with high InhB would range from 0 to 1. To achieve parity between the numerator and the denominator, the hormonal values are first standardised by taking their natural logarithmic values, then examined by taking the differences between the two logged values. When this ratio is standardised using the mean levels of the hormones in the young men, then the distribution centers around zero. Any variation from a normal distribution indicates biological skewing, with the width of the bell curve being a measure of the variation in the population: as per the normal distribution of each individual hormone.

The approaches used in the thesis were based on advice provided by Prof. Herbison, one of the University statistical advisers. Prof. Herbison has retired, and further advice has been obtained from Mr Andrew Gray, the statistical adviser for the Department of Anatomy. Based on this advice, I believe the statistical methods used in the thesis are robust. Much of the data from the thesis has been published, adding support to this view.

With correlative analyses, the unexplained variance must be normal, when Pearson's method is used. There is no requirement for the initial distributions of the variables to be normal. The purpose of a Box-Cox transformation is to normalise the initial distributions, prior to correlation. The initial distributions of some of the hormones examined in the thesis were not normal, but the distributions of residuals for the correlative analysis were normal, or near-normal. For this reason, Prof. Herbison recommended the use of Pearson's method, although all results were also verified by non-parametric analyses, in line with best practice. Box-Cox transformations produce arbitrary alteration to data, which arguably should only be undertaken when no alternative approach exist.

All calculations were performed using Stata Statistical Software, Release 13 (StataCorp).

Chapter 3:
Daily profile of circulating AMH and other testicular hormones

3.1 Introduction

3.1.1 Circadian pattern of endocrine systems

Many hormones exhibit oscillating rhythms. These rhythms respond to changes in the environment, and regulate homeostasis. The pancreas secrete insulin into the portal vein in a pulsatile fashion every four minutes (also known as an ultradian rhythm), and is modulated by the presence or anticipation of food intake (135). The amplitude and the frequency of insulin secretion regulates the metabolism of glucose by the liver (136). Other rhythms exhibit a daily recurring pattern, which is known as a circadian rhythm. Cortisol levels peak in the morning (diurnal pattern) in anticipation of physical stress during waking (137), whilst melatonin level has a secretory burst at night to facilitate sleep (nocturnal rhythm) (138).

3.1.2 Regulation and influences of circadian patterns

The regulation of these circadian and other rhythms are controlled at multiple levels. Most rhythms are centrally mediated by the suprachiasmatic nucleus in the hypothalamus (137), and peripherally within target organs putatively by autonomous pacemaker genes, such as CLOCK and PER genes (139). The interactions between the hypothalamic-pituitary centres and target organs are mediated by positive and negative feedback loops via endocrine and paracrine signalling.

Other endogenous and environmental factors, such as ageing, pathology and the presence of light can modify these effects further. Melatonin secretion, for example, exhibit an age-related decline, and during exposure to nocturnal lighting; with both factors resulting in changes to sleep architecture (140). Similarly, people with Type II diabetes mellitus experience attenuation of their insulin amplitude and pulses into the portal vein that consequently affect hepatic glucose metabolism and hyperglycaemia (136).

3.1.3 Diurnal rhythm of testicular hormones

Amongst the four testicular hormones, the daily profiles for AMH and INSL3 are unknown. In contrast, daily patterns of testosterone and InhB have been well described, with both hormones exhibiting diurnal profiles with morning peak levels (141-149). In clinical practice, there is a tacit assumption that testicular hormonal output is regulated at a pan-testicular level, with physiological and pathological correlation done to morning blood samples of testicular hormones. An alternative theory is that regulation might occur at a cell type level generating common variation between the Sertoli hormones and Leydig hormones [Chapter 1.4]. A third model posits that each hormone is independently regulated, supported by some evidence of partial covariation between diurnal testicular hormones [Chapter 1.4].

3.1.4 Sporadic influences on testicular hormones

Another layer of complexity are sporadic factors that can influence hormonal levels, particularly testosterone. An acute rise in testosterone level can be induced by the onset of REM sleep (150, 151), physical exercise (152-154), interaction with an unfamiliar woman (155), and sexual activities (156), whilst levels are lower with fatherhood status (157), marital status (158), feeding (159), and place of blood collection (160).

3.1.5 Objectives of study

The daily profiles for AMH and INSL3 are unknown. Consequently, there is a lack of understanding of how the endocrine testicular hormones are regulated as a whole, and whether sporadic influences exert common or specific effects on each testicular hormone. This Chapter aims to profile the daily profile for each testicular hormone in healthy young men, and examine for any covariation and sporadic influences between hormones.

3.2 Methods

3.2.1 Study participants

The study was of community-based young men, with no known health issues. Men were recruited from the University of Otago and from the staff of the Dunedin Public Hospital by advertisement. The median age of the men was 26.7 years, with a range of 19.3 to 57.3 years [Table 3.1]. Only one of the men was older than 40 years of age. The men were not taking any regular medications, non-smokers, had no known testicular or pituitary disorders, not obese and not involved in shift work.

3.2.2 Study design

Study 1: blood sampling

Thirteen men had a peripheral intravenous catheter inserted into their antecubital fossa vein at 20:00 for acclimatisation. They were housed either in their own dwelling (n=6) or slept in a communal university hostel (n=7). The sleep location had no effect on the hormone levels, and the data for the two groups were combined. Participants were encouraged to maintain their regular routine and sleeping pattern. A series of 6 mL blood samples were aspirated at the following times: 00:00, 06:00, 09:00, 12:00, 14:00, 17:00 and 19:00 by the author. The 06:00 sampling was fasting and pre-dawn, the 12:00 and 17:00 bloods were pre-meal, with the corresponding post-prandial bloods 2 hours later. Sunrise was approximately 06:30 and sunset was approximately 20:50 during the sampling period. The catheter was flushed with isotonic saline periodically to maintain patency.

Study 2: blood sampling

A second study was conducted to increase the statistical power and validate observations from Study 1. This group consisted of 20 men, 19 new participants and one man from the first study who was re-sampled. Using the methodology

above, blood samples were drawn at 19:00 on day 1 and at 06:00 on day 2, with sunset at 19:00 and sunrise at 07:00 during the second study.

3.2.3 Calculations and statistical analyses

The hormone values for each participant were normalised to their 24-hour mean. This ensures that each participant's value is of equal weight in the statistical tests, by removing the influence of inter-person variation in the absolute level of the hormones [Chapter 2.2].

The number of possible diurnal patterns is large, which limits direct comparison between time points, unless there is an *a priori* hypothesis about the pattern. Study 1 was therefore tested using repeated measures ANOVA, with pairwise comparison as the post-hoc test. Study 1 detects overt diurnal variation, as occurs with cortisol and testosterone, but lacks the power to detect subtle variation. Hence, the second study was designed to test a specific diurnal pattern, with the 06:00 and 19:00 samples being compared using a paired Student *t*-test.

Covariance between the testicular hormones were tested using mixed model analyses with the hormones as independent continuous variables, and time points as categorical variables (161).

3.3 Results

3.3.1 Baseline characteristics

The baseline characteristics of participants in both studies are presented in Table 3.1. The 09:00 values for testosterone, InhB, and gonadotropins were within the normal range of the local clinical laboratory for morning sample. Only one individual had a testosterone level that was marginally low at 8.7 nM (9-38 nM). This man was not obese, and had proven fertility. Physiological ranges for AMH and INSL3 have not been established in men. The AMH values of all men in this study were within a reference biochemical range of local men with defined health status [Chapter 4.3].

All men in Study 1 exhibited a normal daily pattern of cortisol [Fig 3.1D], establishing that their circadian rhythms were intact. LH had a significant diurnal variation with higher values in the nocturnal and early morning periods, and subsequently trending towards an evening nadir [Fig. 3.1C, p=0.032 ANOVA]. FSH, in contrast, did not vary significantly between time points [Fig. 3.1C, p=0.36 ANOVA]. Similar results were obtained for LH and FSH in Study 2 comparing 06:00 and 19:00 values in 20 men (LH 6:00 4.7 ± 0.5 IU vs. 19:00 3.7 ± 0.3 IU, p=0.032 paired Student's *t*-test; FSH 6am 4.1 ± 0.5 IU vs. 7 pm 4.0 ± 0.4 IU, not significantly different). These findings were consistent with reported gonadotropins daily profiles (162, 163).

Table 3.1: Characteristics of participants in both diurnal studies

	Study 1	Study 2	Lab ref range
Sample size	13	20	
Age (years)	28.7 ± 9.5	25.8 ± 3.2	
AMH (pM)	48.2 ± 22.4	57.6 ± 23.5	N/A
INSL3 (ng/mL)	1.7 ± 1.5	2.4 ± 0.9	N/A
T (nM)	17.3 ± 6.2	20.2 ± 9.2	9 – 38
InhB (pg/mL)	178.9 ± 51.9	187.3 ± 68.0	30 - 320
FSH (IU)	5.2 ± 1.8	4.7 ± 2.0	2 - 12
LH (IU)	2.9 ± 1.9	3.7 ± 1.4	2 – 9
Cortisol (nM)	377.7 ± 119.4		250 – 800

The data are the mean ± standard deviation of the 0900 time of day values for study 1 and the 0600 value for Study 2.

3.3.2 Sertoli cell hormones

There was no statistical variation in AMH across the day in study 1 (p=0.48 ANOVA), indicating the absence of a consistent and overt diurnal profile. However, AMH levels were moderately lower in the nocturnal phase, with a nadir at 06:00, and a subsequent rise in the hours after waking [Fig. 3.1A and 3.2A]. The veracity of this subtle variation was tested in Study 2, which confirmed that the 06:00 levels were slightly lower on average (4.9%, p=0.004, paired Student's *t*-test), with the inter-person variation encompassing a range between -7.9% and 18.1% [Fig. 3.3].

In contrast, InhB exhibited an overt diurnal pattern with significant variation throughout the day [Fig. 3.1A, p<0.0001]. The InhB values peaked at 09:00 and then diminished to a 19:00 nadir, with an average total decrease of 26% during this period. This diurnal pattern was observed in 11 of the 13 participants, with the levels of InhB in the other two men being relatively stable throughout the day [Fig. 3.2B].

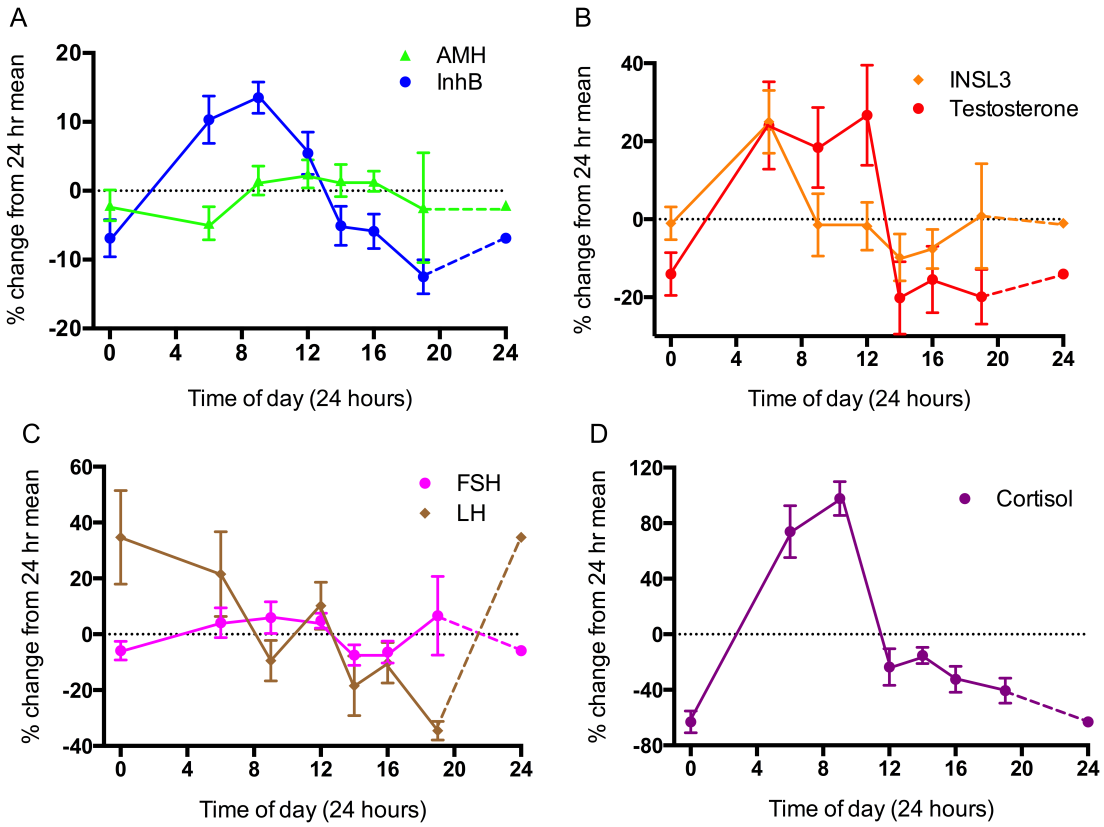

Figure 3.1. Study 1: diurnal pattern of hormonal expression.

Each individual's hormone levels were normalised to that individual's mean across the entire sample period. (A) Sertoli cell hormones: InhB (blue circles) and AMH (green triangles); (B) Leydig cell hormones: testosterone (red circles) and INSL3 (orange diamonds); (C) Gonadotropins: LH (brown diamonds) and FSH (pink circle) and (D) Circadian marker: Cortisol. The single 0:00 data point is plotted at both 0:00 and 24:00 with the dashed line used to indicate this extrapolation. The data are the mean ± the standard error of 13 men.

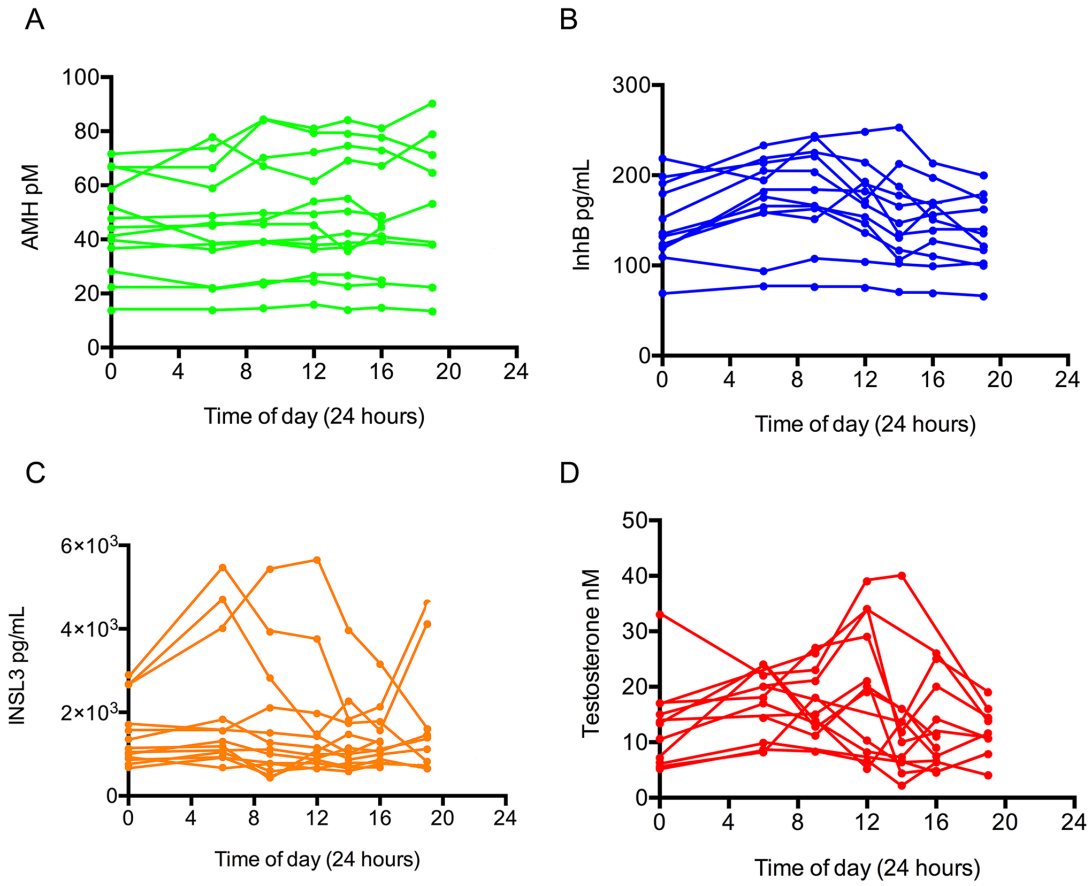

Figure 3.2. Study 1: Daily trend of serum testicular hormones from 13 healthy men plotted individually.

(A) AMH, (B) InhB, (C) INSL3 and (D) testosterone. The 00:00 data points are plotted at both 0:00 and 24:00, with the dashed line used to indicate this extrapolation.

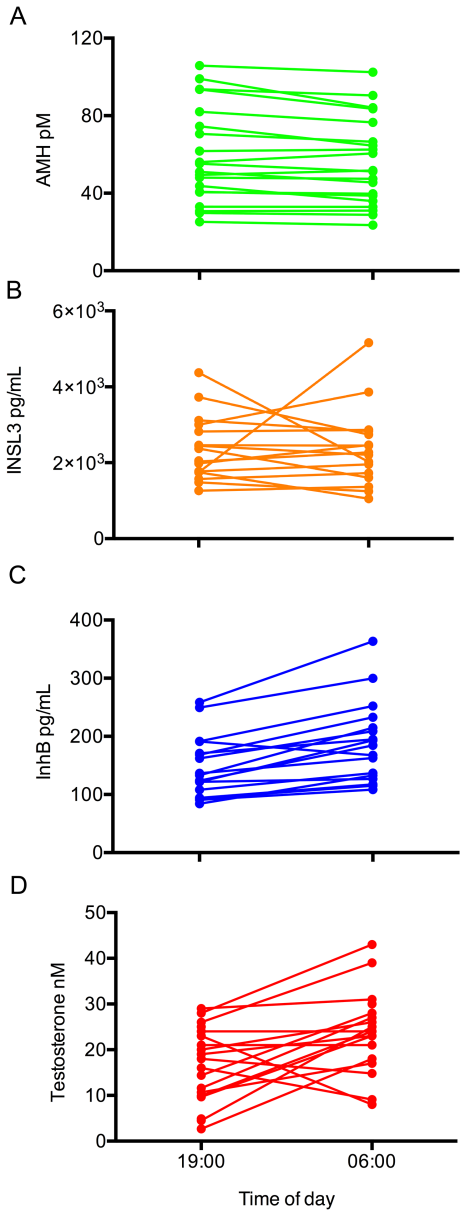

Figure 3.3. Changes in serum testicular hormonal concentration between 19:00 and 06:00 (Study 2).

(A) AMH, (B) INSL and (C) InhB and (D) testosterone levels were analysed in 20 men. AMH, on average declined by 4.9%, (p=0.004, paired Student t-test), whereas the levels of InhB and testosterone increased on average by 30% (p=0.000) and 38% (p=0.011) respectively. There was no significant change in the levels of INSL3, on average (p=0.78).

3.3.3 Leydig cell hormones

INSL3 was the least variable testicular hormone with no significant variation observed across the study population [Fig. 3.1B, p=0.13 ANOVA]. The moderate spike in INSL3 observed at 06:00 was predominantly caused by variable levels in 3 individuals with no consistent circadian pattern [Fig. 3.2C]. When each man's values were normalised to his average value for the day, the standard deviation for all the data points was 26%. There was also no significant difference on average in INSL3 levels in Study 2 (p=0.78 paired Student's *t*-test), although 4 of the 20 men had non-concordant values at 06:00 and 19:00, but without consistent direction; two had higher values at 06:00 and two had lower [Fig. 3.3B].

Serum testosterone levels in Study 1 were consistent with the established diurnal profile (148), with a peak morning value and a nadir concentration in the evening [Fig. 3.1B, p=0.04 ANOVA]. There was an average decrease of 47% between the 06:00 and the 19:00 nadir samples. This circadian pattern was replicated in Study 2 with an average 38% increase between 19:00 and 06:00 the following day [Fig. 3.3C, p=0.011, paired Student's *t*-test]. The average amplitude for the testosterone diurnal variation was larger than that of InhB (47% vs. 26%, respectively), but with less statistical confidence because the testosterone rhythms were highly variable between individuals.

3.3.4 Hormonal covariation

AMH, INSL3 and testosterone did not covary to each other when comparing the entire groups and each individual. For example, the individuals with the most overt changes in AMH levels did not have the largest changes in testosterone. Similarly, the individuals with some variation in their levels of INSL3 had unremarkable diurnal variations in testosterone and InhB, relative to other men. The time-point-to-time-point variation in InhB partially correlated to testosterone only ($p=0.001$, mixed model covariation) and not to either AMH or INSL3 [Table 3.2].

Table 3.2: Covariation of the diurnal patterns of the hormones

	InhB	AMH	Testosterone
InhB	-	0.06 (p=0.48)	1.26 (p=0.001)*
AMH	0.06 (p=0.48)	-	-0.30 (p=0.65)
Testosterone	1.26 (p=0.001)*	-0.30 (p=0.65)	-
INSL3	0.09 (p=0.049)*	0.03 (p=0.43)	0.07 (p=0.68)

The covariation between time points for each of the testicular hormones were calculated for 20 men using mixed model analysis, and expressed as the covariation coefficient (p value). Note that the data is normalised to the daily mean, and is therefore independent of the absolute levels of the men's hormones. * indicates statistical significance.

3.4 Discussion

3.4.1 Diurnal pattern of testes is regulated at level of the hormone

AMH has a subtle nocturnal variation with an evening peak whereas INSL3 exhibits no overt circadian pattern. Hence, the Sertoli and Leydig cells each secrete one hormone with minimal variation during the day (AMH and INSL3) and another hormone with an overt diurnal pattern (InhB and testosterone), indicating that the diurnal variation operates at the level of the hormone and is not a universal property of the endocrine cell type.

The circulating level of a hormone is a product of its synthesis, secretion and clearance. Testosterone and InhB, which had the most covariance, have similarly short half-lives in blood, ranging from 23 minutes to 45 minutes (164-166). AMH, in contrast, has a long half-life that is not fully characterised in men. In women and bulls the half-life of AMH exceeds one day (59, 167). Consequently, the presence of a subtle change in the levels of AMH between 06:00 and 19:00 [Fig. 3.3] may represent a larger diurnal variation in either the production or the secretion from the testes, with the magnitude of the change in serum levels being dampened by its slow clearance.

The synthesis of AMH is putatively decreased by testosterone [Chapter 1.4.3]. The diurnal decrease in AMH coincided with a diurnal rise in testosterone, raising the possibility that the variation in AMH is secondary to the larger diurnal variation in testosterone. However, both AMH and testosterone cycles vary in magnitude and direction between men. Consequently, if the daily pattern of AMH levels is driven by testosterone then the person-to-person variation in the two hormones should associate, which was not the case. This suggests that testosterone is not a main driver of the daily variation in AMH levels.

The diurnal rhythm of InhB and testosterone partially covaried, despite being derived from different testicular cells, and has been reported in some studies (141, 142). The pan-testicular effect suggests a common influence driving the

circadian rhythm of the testes. One putative candidate is gonadotrophin releasing hormone, by driving the gonadotropins FSH and LH. The basal secretion of InhB is mediated by FSH, and testosterone similarly is influenced by LH. LH is related to testosterone levels but only during the early hours of the morning whilst asleep (146, 168), with the total daily relationship between gonadotropins and testicular hormones being independent of each other (142). Human chorionic gonadotropin (HCG) mimics the effect of LH as a stimulant to Leydig cells in secreting testosterone. Men who have low levels of testosterone secondary to low gonadotropins can reestablish their diurnal pattern of testosterone from a single injection of HCG (169). Taken together, gonadotropins may play a role in the generation of testicular diurnal rhythm, particularly with testosterone, but not in a direct temporal relationship.

Intra-testicular elements have not been shown to regulate the circadian pattern of testicular hormones. Testicular autonomous pacemaker genes, such as PER and CLOCK genes similarly do not generate circadian patterns of expression, unlike other peripheral organs such as the kidneys and liver (139, 170) and therefore unlikely to regulate diurnal rhythms of the testes.

3.4.2 INSL3 as a biomarker for Leydig cell function

Testicular hormones are often used as surrogate biomarkers of their respective testicular cell types. InhB exhibit a more marked diurnal pattern than AMH, making it a less reliable biomarker for Sertoli cells. In a similar context, INSL3 is an emerging biomarker for hypogonadism, with the greater day-to-day consistency of INSL3 over testosterone being advantageous in this context (10, 33). The absence of diurnal variation in INSL3 further supports the use of INSL3 as a biomarker for Leydig cell function. The overall intrapersonal variation in INSL3 levels between points was 26%, which is similar to the 21% day-to-day variation reported by Ivell et al (33). These values include variation from the ELISA, indicating that the level of sporadic variation in INSL3 levels is less than 10%.

Testosterone is known to exhibit sporadic variation due to multiple environmental determinants. INSL3 levels within a male is shown to be relatively invariant for the majority of the men studied, suggesting that these sporadic influences have no influence on INSL3 levels.

A minority of men in Study 1 had very high concentrations of serum INSL3, which was also observed in Study 2. The combined data from the two studies showed a right skewed distribution [Fig. 2.1] that was significantly different from normality (SK test skewness 1.01, p=0.03) with a range that encompassed an order of magnitude. This is the first longitudinal study examining INSL3 in young men, and the current body of literature on INSL3 is small. However, two large cross-sectional studies on INSL3 in men also found similarly right skewed distributions in a pattern not different to the current study [Fig. 2.1], with similar ranges (30, 171). One possible interpretation for the phenomenon common to all three studies is that INSL3 is released in a pulsatile manner similar to LH (ultradian rhythm), or affected by sporadic influences like testosterone. The clearance of INSL3 is unknown.

3.4.3 Technical issues

This study had no *a priori* assumptions of daily pattern of AMH and INSL3, and hence took into account several factors for study design. Firstly, all the men in the first study were sampled in the same season to remove any potential effect of seasonality. Similarly, the timing for blood tests coincided with various social and physiological processes that are known to affect testosterone, such as pre-dawn sleeping fasting blood test (0600), post prandial bloods (0900, 1400, 1900), and midnight sleeping (0000). No restrictions were placed on each participant's daily habits, including being in their place of abode to mimic usual rhythm for the participants. Testosterone level can be confounded by sporadic influences such as the presence of a female and sexual activities, and it is unknown if this phenomenon also affects the other testicular hormones. The study therefore required blood sampling to be performed by the male author, and strict instructions given to participants to abstain from any sexual activities

during the sampling period.

The first study observed a small nocturnal rise, with invariance in INSL3 levels overnight. The second study was therefore conducted to confirm this finding in another cohort of men between evening and the following early morning, which validated the initial results with statistical significance.

Both studies were designed to examine for circadian rhythms, and therefore any additional ultradian patterns particularly for AMH and INSL3 were unable to be profiled, due to frequency of sampling that would be required. Gonadotropins secretions are pulsatile, and levels within an individual would vary considerably with a frequency that is shorter than the time intervals for the testicular hormones. This therefore precludes any covariant analysis between the pituitary and testicular hormones.

The study also did not examine for the effect of ethnicity, seasonality, diet, sexual activities and other environmental influences. The number of participants recruited in both studies was of similar magnitude to other circadian observational studies of this nature (141-149).

One participant, out of the total sample size of 28 (3%) had peak morning testosterone level that was marginally below the local laboratory reference range. Reference ranges are usually defined by the levels found in the middle 95% of a normal reference population. The remaining 5% of these normal people will therefore have values that fall outside of this range. This is likely to be the case with our participant, who did not have any features of androgen deficiency (149, 172), and consequently was included in the data analyses.

3.5 Conclusion

AMH and the other three testicular hormones exhibit distinct daily profiles in the blood of young men, indicating that the regulation of circadian cycles are at a hormonal level, rather than cell type or pan-testicular level. Individual variations exist for all hormones, which infers sporadic influences that are yet to be determined. The physiological significance of this finding is unclear due to the paucity of information relating to the non-reproductive role of protein testicular hormones. This study however guides blood sampling to be ideally performed in the morning for testosterone and InhB, with AMH and INSL3 levels being stable throughout the day enabling sampling to be done at any time.

Chapter 4:
Circulating AMH levels in older men and women, in comparison to younger men

4.1 Introduction

This thesis aims to explore the role of circulating AMH level in men, an area that currently lacks any data. At the commencement of this thesis, AMH research in males had predominantly focussed on development and childhood. Consequently, AMH levels were not known beyond early adulthood [Chapter 1.5.2].

AMH levels vary between boys (41). Most males exhibit a decline in their AMH levels during the pubertal transition, resulting in post-pubertal AMH on average being an order of magnitude lower than boyhood levels [Chapter 1.5.2]. A recent study has found that post-pubertal AMH is predominantly genetically determined (173). Information regarding any variability in men that is currently unknown will therefore form the basis for examination of interaction between AMH with other testicular hormones [Chapter 5], as well as association between AMH and specific traits [Chapter 7].

This Chapter will also examine AMH levels in older men. Many hormones undergo age-related decline, with the classical example being growth hormone (174, 175). InhB levels however are thought to be stable throughout the adult male lifespan (176-178). Both InhB and AMH have been utilised separately as biomarker for Sertoli number and function. AMH and InhB are independently

regulated with respect to their daily levels [Chapter 3.3.2], and this Chapter will examine if both hormones remain independent across the lifespan, or if both hormones are linked due to changes in Sertoli cell number or function.

AMH levels remain largely unchanged over the course of 24 hours, as shown in Chapter 3.3.2. This allows the measurement of AMH in a cross-sectional study design, to provide population level data as a basis for comparison of AMH levels in different population groups. To enrich the dataset, sera from three cohorts independently recruited were assayed for their Sertoli hormone level to profile AMH across lifespan. The focus of this thesis is on the older male; hence the design of this study has a bias towards recruitment of older males.

In contrast to men, AMH levels has been profiled extensively in women, which decline in parallel to age-related diminishment of their ovarian reserve [Chapter .5.2]. The normal curves for AMH in women however do not extend significantly beyond the age of menopause, with older women presumed to have no circulating AMH [Chapter 1.5.2]. However, in some primates, AMH-producing granulosa cell clusters are a normal feature of the aged ovary, with the clusters increasing in size after reproductive senescence (179). A small group of older women were therefore examined as part of this thesis, to determine whether circulating AMH in the elderly is a male-specific hormone, and reported in the second part of this Chapter.

Chapter 4A: Circulating AMH levels in men

4A.2 Methods

4A.2.1 Participants

Serum samples were collected from 657 men aged from 19 to 93 years, with a focus on elderly men (n=522). The participants were drawn from three distinct cohorts, with the Otago cohort recruited by the author, and the Vascular and LiLACS (Life and Living in Advanced Age: A Cohort Study in New Zealand; Te Puawaitanga o Nga Tapuwae Kia Ora Tonu) NZ cohorts recruited by other research groups. A non-fasting 5 mL venous blood sample was obtained in the antecubital vein between 7 am and 3 pm.

The Lower South Regional Ethics Committee (NZ) approved the studies on the Vascular cohort and the older men subgroup from the Otago cohort, whilst the younger men from the Otago cohort was approved by the University of Otago Human Ethics Committee. The LiLACS NZ cohort was approved by the Northern X Regional Ethics Committee (NZ). The ethical approvals permitted the assay of sera in collaborative studies collected from the Vascular and LiLACS NZ cohorts.

4A.2.1A Otago cohort

The Otago cohort was a cross-sectional study of 99 community dwelling men aged 70 or older, with 124 younger men as a comparison. The men were predominantly Caucasian and resided in Dunedin, a small university city in New Zealand. Men were excluded from the study if they had a history of testicular tumour or trauma, orchiectomy, undescended testes, self-reported infertility, chemotherapy or abdominal or pelvic radiation therapy. None of the men were on anti-androgen medication or steroid medications.

All the men in this cohort were recruited from the community via advertising flyers. The older men of the cohort (aged 70 years and older) were recruited

from community groups and social clubs, including Age Concerns meetings, Probus Club, local bowling clubs, University of the Third Age (University of Otago), local church congregations, and the Dunedin Phoenix club. Some older men joined the study from viewing advertisements placed in the University, the local hospital foyer, and a local community newspaper. A small subgroup of older men was recruited from the hospital's Older Person's Health community outpatient rehabilitation classes. These men were living in their own homes, able to walk without assistance from another person, but had a range of physical, psychiatric or cognitive limitations requiring rehabilitation. All men recruited were able to provide informed consent to participate in the study.

The younger men aged from 19 years to 69 years were recruited from various facilities and work places in the Dunedin metropolitan area. These included students and staff from the University of Otago and local hospital, civil service employees, office based private sector employees, workers from light and heavy industries, and a local karate club. None of these men were shift workers, and all had no known history of medical issues or medications that would affect the pituitary-gonadal axis.

4A.2.1B Vascular cohort

The vascular cohort was a separate Dunedin cohort comprised of 153 community dwelling men aged 54–93 years, with no history of cardiovascular disease. These men were recruited from community advertisements and excluded if they have features of peripheral arterial disease and carotid stenosis. This was defined as having a carotid stenosis of 50% or greater on Doppler ultrasound scan, and/or an arterial pressure ratio at the ankle compared to the brachial artery of less than 0.9, or greater than 1.3. These men were recruited and blood sampled by the Vascular Research Group from the University of Otago. Permission was granted to access their biobank for the men's sera. None of the participants from the Otago cohort were duplicated in the Vascular cohort.

4A.2.1C LiLACS NZ Cohort

Sera from 257 elderly men were analysed from the LiLACS NZ Research Group. This is an ongoing epidemiological study examining factors that lead to successful aging in older New Zealanders. A cohort of 937 community-dwelling men and women were recruited in the East Coast (Bay of Plenty and Rotorua) of the North Island, from the electoral roll registry, with methodology described elsewhere (180). The inclusion criteria were primarily based on age and ethnicity: Māori aged between 80–90 years old, and non- Māori aged 85 years old were recruited, with a response rate of 65% from the male cohort. Study participants had varying health and physical performance status measured, with the 257 men examined being those that had consented for their sera to be used for collaborative research. None of the participants reported a history of orchiectomy.

4A.2.2 Hormonal analysis

Sera were assayed for their AMH and InhB levels as described in Chapter 2. The AMH reported and calculated here are based on the values obtained prior to the field notice from Beckman Coulter [Chapter 2.1.5] for consistency in comparing between the three distinct cohorts.

4A.3 Results

4A.3.1 Description of Sertoli hormones in the Otago cohort

4A.3.1A AMH

The levels of AMH were highly variable between men, with a distribution that was positively skewed both in men younger than 50 years [Fig. 4.1A, SK Test 1.4, $p<0.0001$, n=111], and in those older than 50 [Fig. 4.1B, SK Test 2.1, $p<0.0001$, n=112]. All younger men under 50 years had AMH levels greater than 6.6 pM. There was no correlation between AMH level and age in these younger men ($R^2=0.03$, p=0.10, n=111), after which there was a change in the profile of AMH levels [Fig. 4.2A and B]. In men over 50 years of age, there was a progressive and significant decline in the geometric mean [Fig. 4.2B, $p<0.0005$ ANOVA], and the median AMH in older men was nearly half that of younger men [Table 4.1]. A quarter of older men had AMH levels below the cohort's reference range generated from younger men's values [Table 4.1]. There was a concurrent increase in the variability of AMH levels between older men: 5 men had levels below the limit of detection, but none of the older men had AMH levels above the upper reference range. Consequently, the coefficient of variation increased with age, from 50% in those younger than 30 years old (n=33), to 105% in men over 80 years (n=36) [Fig. 4.2B].

4A.3.1.B InhB

The profile of InhB across the lifespan was similar to that of AMH. InhB was less variable between individuals than AMH [cf. Fig. 4.2A, 4.2C], and was normally distributed in both younger and older men subgroups [Fig. 4.1C,D]. An age-related change in InhB was only observed in older men. As with AMH, this involved a diminishing geometric mean with age, with a concurrent increase in the coefficient of variation [Fig. 4.2D]. 21% of the older men had levels of InhB below the reference [Table 4.1].

Table 4.1 Description of the Otago cohort

	< 50 years	≥ 50 years	Entire cohort
Numbers	111	112	223
Mean age years	36.4	75.8	56.3 (range 19.3 – 89.7)
Mean AMH (sd) pM	31.4 (18.3)	17.1 (14.3)	24.0 (17.7)
Median AMH (range) pM	29.3 (6.6 – 91)	13.2 (0.0-82.8)	32.1 (0.0-91)
Ref range AMH pM	8.2 – 85.6		
% below ref range	1.8%	25%	13.5%
Mean InhB (sd) pg/mL	174 (62)	108 (59)	141 (69)
Median InhB (range) pg/mL	175 (32-364)	110 (0-275)	134 (0-364)
Ref range InhB pg/mL	61.4-311.2		
% below ref range	1.8%	21.4%	11.7%

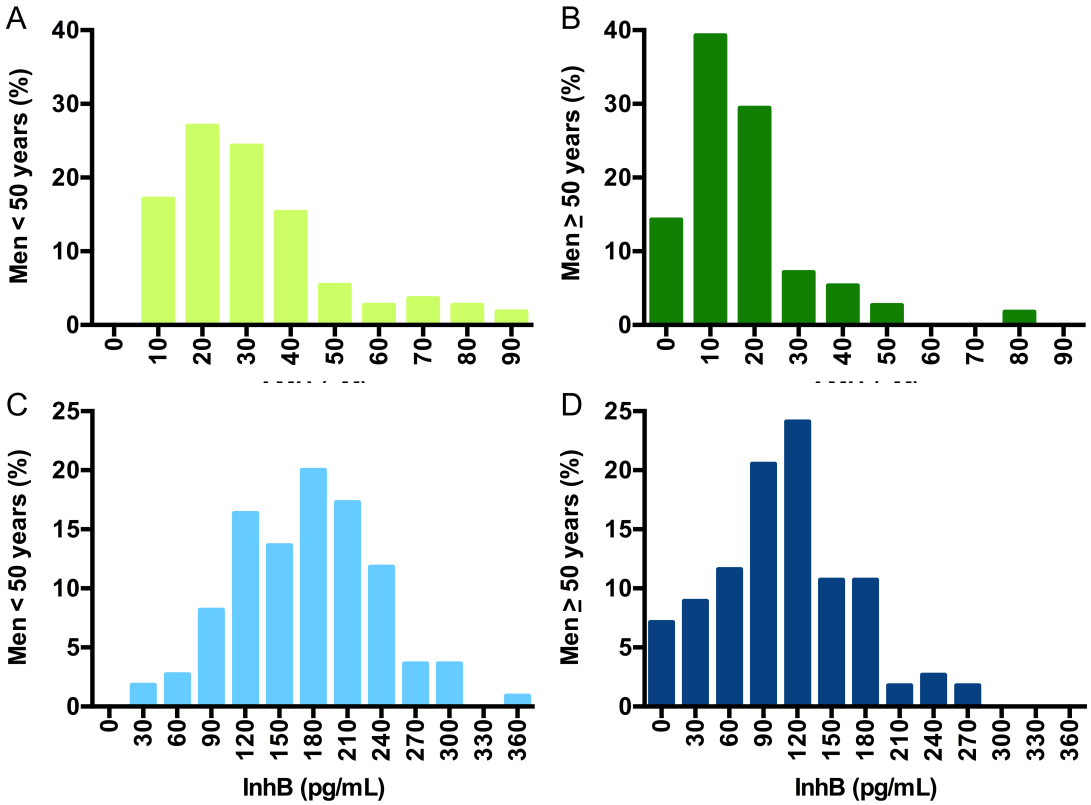

Figure 4.1: Distribution of Sertoli hormone levels in men.

A: the distribution of serum AMH in the young subset of community dwelling men in Otago (n = 111, 19–50 years), B: distribution of AMH in the older subset in Otago (n = 112, 50–90 years). Both distributions for AMH were positively skewed (skewness 1.4 for younger and 2.1 for older subgroups). C: distribution of serum InhB in the young subset of community dwelling men in Otago (n = 110, 19–50 years), D: distribution of InhB in the older subset in Otago (n = 112, 50–90 years). Both distributions for InhB were normally distributed (skewness 0.2 for younger and 0.3 for older subgroups).

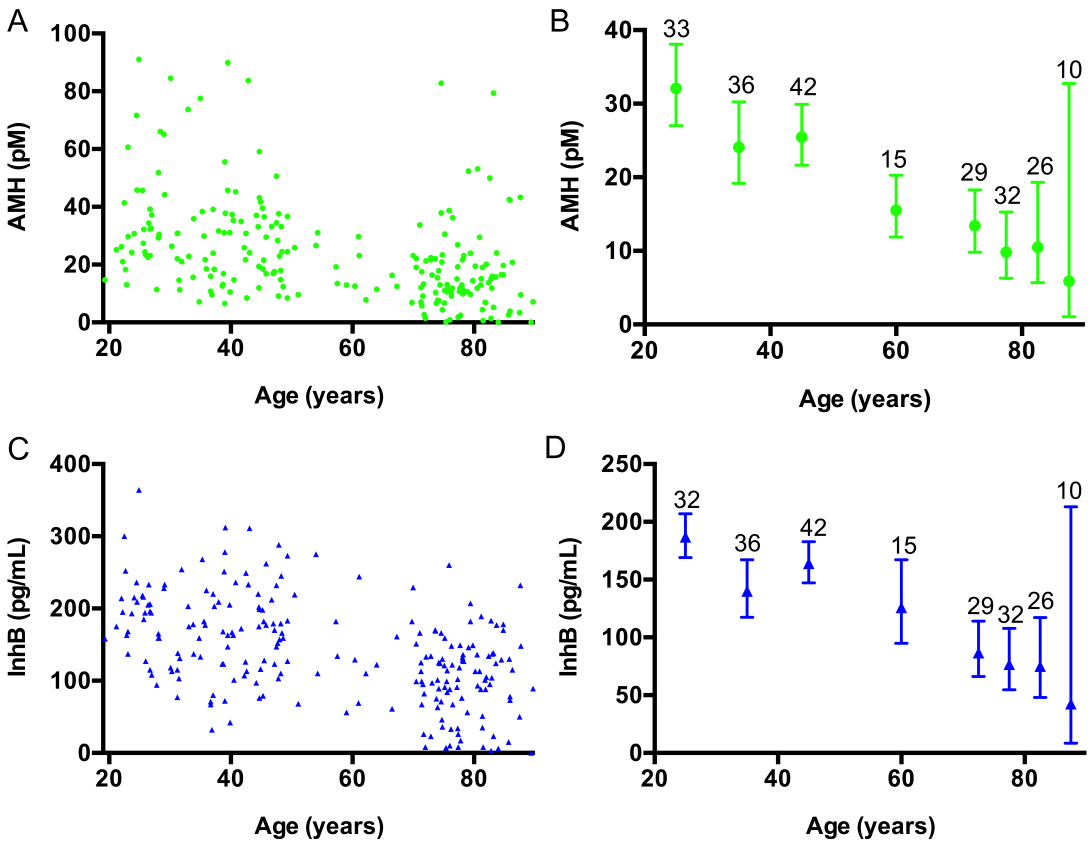

Figure 4.2: The levels of serum Sertoli hormones in 223 community dwelling men (Otago cohort) are illustrated.

A: each circle represented an individual's AMH value. B: the geometric mean and 95% confidence interval for AMH is indicated for various age groups. The levels of AMH in the older men (80–85 years) were significantly less than those in the younger men (19–30 years) ($p = 0.001$). C: each triangle represented an individual's InhB value. D: the geometric mean and 95% confidence interval for InhB is indicated for various age groups. The young men were grouped by decade (20-29.9; 30-39.9;40-49.9) whereas the elderly men were group by hemi-decade (70-74.9;75-79.9; 80-84.9; 85-89.9). Only limited data was collected from men of intermediate age, precluding further subdivision of the 50-69.9 year-old group.

4A.3.1.C Sertoli hormonal balance

The relative levels of AMH and InhB varied between individuals across the cohort, with a minority of older men having more InhB than AMH [Fig. 4.3A]. All men (n=5) with undetectable levels of one hormone simultaneously had undetectable levels of the other [Fig. 4.3C], with all of these men being over 75 years old. Approximately half of the men with AMH levels below the reference range also had InhB levels below its reference range, with this observation replicated when examining low levels of InhB. Consequently, the levels of the two hormones were not completely independent as a Pearson's linear regression between them was R^2=0.22 [Fig. 4.3C].

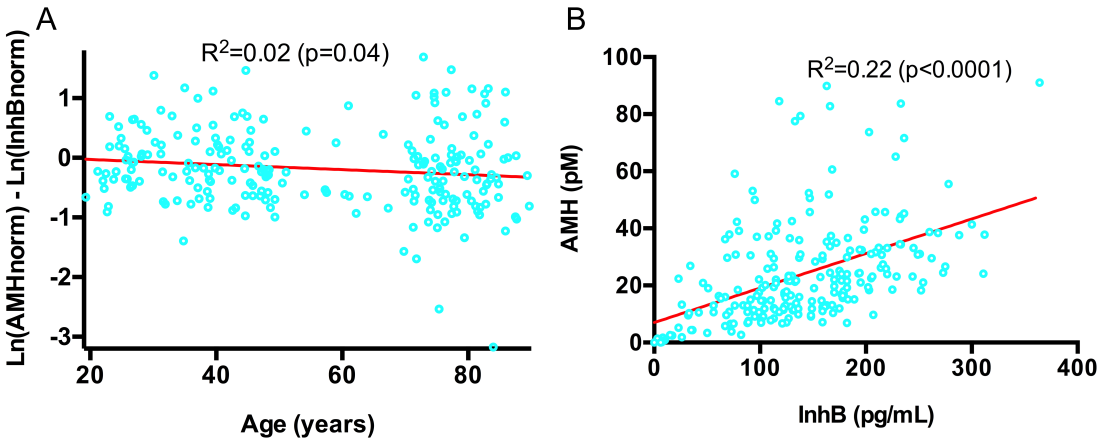

Figure 4.3: The relative ratio of Sertoli hormones for the Otago cohort.

Each cyan circle represents an individual, across age in A. B: The relationship between AMH and InhB is illustrated, with Pearson's linear correlation $R^2 = 0.22$ ($p<0.0001$).

4A.3.2 Comparison of AMH with other cohorts

The Otago cohort was then compared with two other independently recruited cohorts, to determine generalisability of the findings.

The first comparison was between the Otago cohort described above, and a second cohort recruited from the same geographical region, but selected for the absence of cardiovascular disease (Vascular cohort). The average level of AMH in the Vascular cohort (n=153) was higher than the age-matched participants from the Otago cohort [Fig. 4.4, mean difference AMH 9.5 pM, p<0.0001, Student's *t*-test], but were no different to the younger portion from the Otago cohort [Fig. 4.4A, p=0.06]. Two participants from the Vascular cohort had AMH levels above the upper reference range in contrast to none of the aged-matched Otago cohort. Consequently, only 8.5% of the men from the Vascular cohort had AMH levels below the reference range. A minority of these men had undetectable levels of AMH, in similar prevalence to the older Otago cohort [Table 4.2]. In contrast, the difference in InhB values was less pronounced between the Vascular cohort and the aged matched Otago cohort (mean InhB difference 16 pg/mL, p=0.054, Student's *t*-test), with 35.9% of the Vascular cohort having InhB values below the reference range.

The older portion of the Otago cohort was then compared to the LiLACS cohort (n=270), a geographically distinct group of men in the East coast of North Island with a significant component of Māori ethnicity. The age-matched AMH values between the two cohorts were similar [Fig. 4.4B, p=0.14]. The coefficient of variation in the LiLACS cohort was similar to the older Otago subgroup (102% vs. 96%), despite 17% of men in the LiLACS group having undetectable AMH levels, in comparison to 8% of the Otago cohort [Fig. 4.4B]. One LiLACS participant had atypically high AMH level above the upper reference range. InhB values were similar between the two cohorts (p=0.51). 34% of the LiLACS cohort contain Māori men, and by design were older (mean age difference 2.3 years, p<0.0001). AMH values between Maori and non- Māori men were similar (p=0.41) but Māori men had lower InhB values (mean InhB difference 28 pg/mL,

p=0.0041). One Māori man and 8 non- Māori men had InhB levels above the upper reference range [Fig. 4.4D].

When the three cohorts were combined, older men displayed a larger divergence in the ratio of AMH and InhB, in both the positive and negative directions. The vascular group had a prominent AMH bias, whereas the LiLACS group had an InhB bias [Fig. 4.5]. There was no racial difference in the Sertoli hormonal balance in the LiLACS cohort (p=0.20).

Table 4.2 Comparisons of AMH values between 3 different cohorts

	Otago	Vascular	LiLACS NZ
Numbers	223	153	270
Mean age (range) years	56.3 (19-90) years	71.7 (54-93) years	85.3 (81-91) years
AMH Mean (sd) pM	24.0 (17.7)	26.7 (16.6)	13.7 (14.0)
AMH Median (range) pM	32.1 (0-91)	22.7 (0-103)	11.4 (0-118)
% below AMH ref range	13.5%	8.5%	40.7%
InhB Mean (sd) pM	141 (69)	94 (61)	109 (80)
InhB Median (range) pM	134 (0-364)	81 (6-302)	101 (2-379)
% below InhB ref range	11.7%	35.9%	26.7%

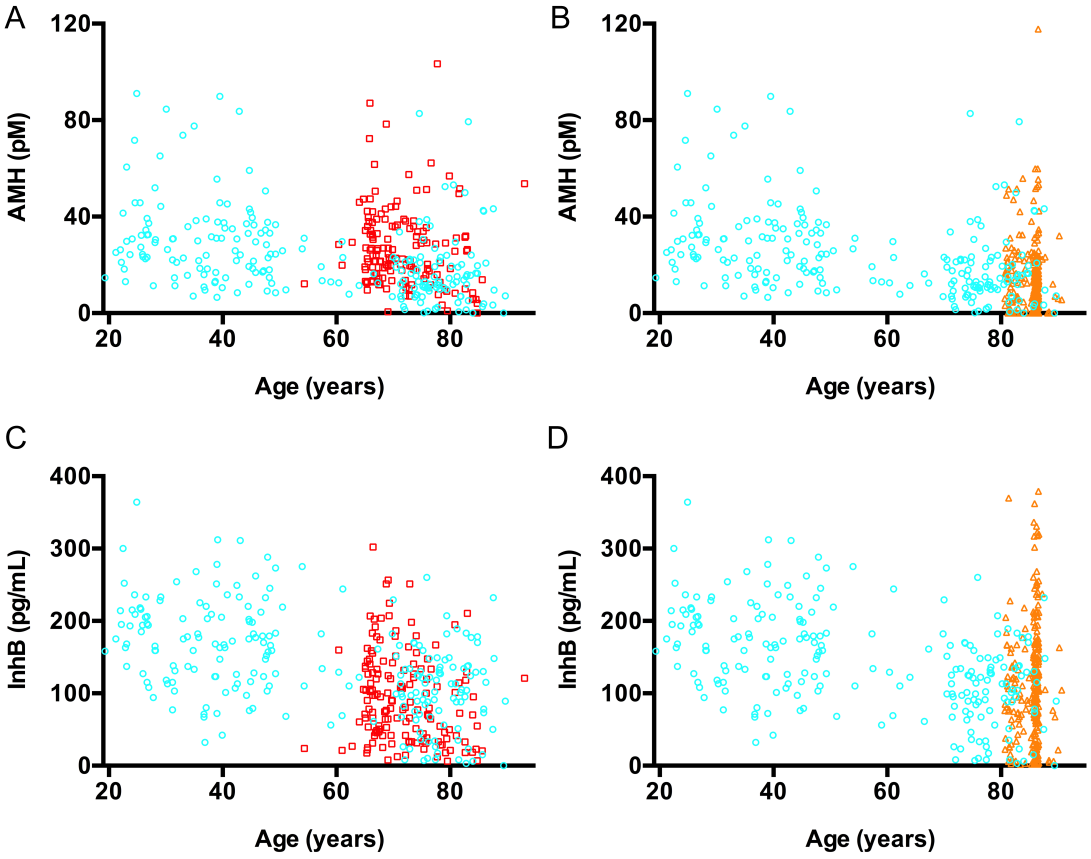
A
B
C
D
AMH (pM)
InhB (pg/mL)
Age (years)
0
40
80
120
100
200
300
400
20
60

Figure 4.4: A comparison of Sertoli hormone levels between Otago cohort and 2 other New Zealand cohorts.

The Otago cohort (223 community dwelling men in Dunedin) is illustrated with cyan circles; the Vascular cohort (153 men in Dunedin selected for their absence of cardiovascular disease) with red squares and the LiLACS NZ cohort (270 men from a distinct geographical area to Otago) with orange triangles. Each symbol represents an individual. A: The Vascular cohort had a higher mean AMH value than the aged matched portion of Otago cohort ($p<0.0001$), but were not different to the younger subset (19–50 years), $p = 0.06$. B: The LiLACS NZ cohort showed similar aged related decline in AMH ($p = 0.14$) when compared to the aged matched subset of the Otago cohort, whilst being different to the younger subset $p<0.0001$ (19–50 years).C: The vascular cohort had similar InhB levels to the aged matched subset of the Otago cohort ($p = 0.13$), but lower than levels than the younger Otago subset (p,0.001). D: The LiLACS NZ cohort showed similar aged related decline in InhB ($p = 0.76$) when compared to the aged matched subset of the Otago cohort, whilst being different to the younger subset $p=0.001$ (19–50 years).

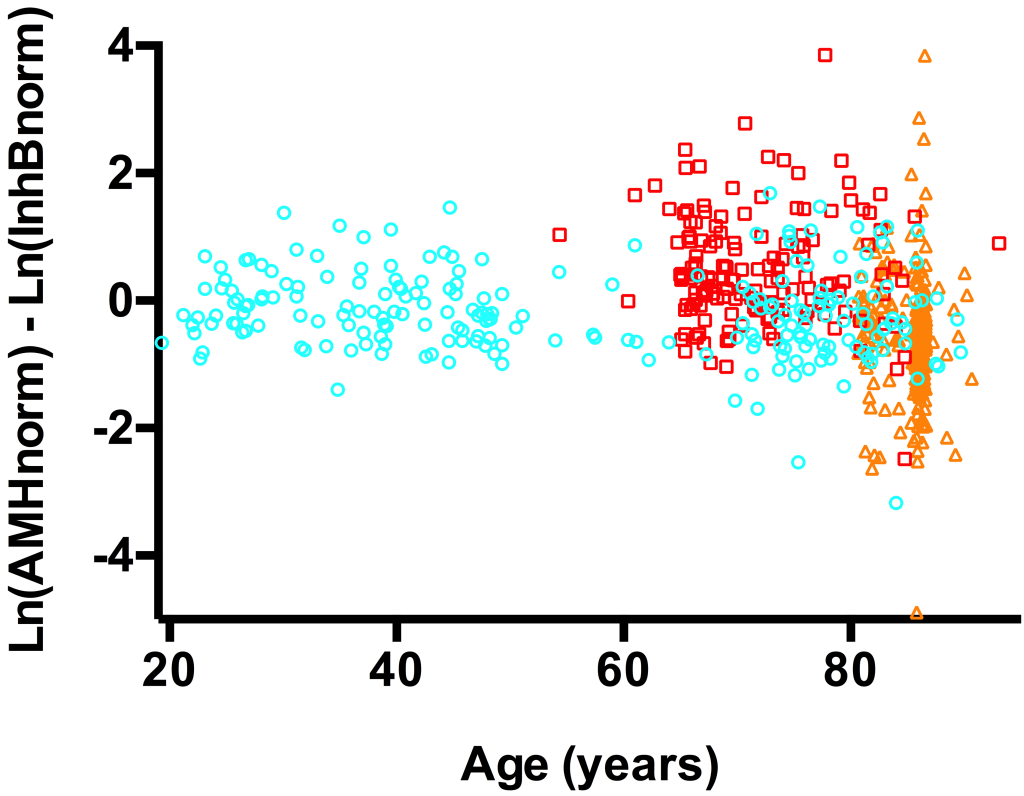

Figure 4.5: The ratio of AMH and InhB in the cohorts of community dwelling men.

The Otago cohort is represented by cyan open circles (n = 223), the vascular cohort by red open squares (n = 153) and the LiLACS NZ cohort by orange open triangles (n = 270). Each open symbol represents an individual. An increasing divergence was seen in the older age groups. The vascular cohort had an AMH bias.

4A.4 Discussion

4A.4.1 Levels of AMH in adult males across lifespan

At the commencement of this PhD, young men were known to have circulating AMH levels. This Chapter extends the observation to show that circulating AMH persists into the very old. The range of AMH values in this Chapter are broadly consistent with subsequently published data of AMH levels in an unselected cohort of younger men (41).

All men from the three cohorts studied had AMH levels at least an order of magnitude lower than boyhood levels, with the highest values measured (118 pM) being less than half of the lowest range in boys (41, 62). Most (41), but not all males (68) exhibited a decline in AMH levels during the pubertal transition. Seminal fluids in men contain AMH (181), at concentrations higher than serum level (182), although not consistently. One possibility to explain this discrepancy is that for some men, AMH becomes preferentially secreted into the seminiferous tubular lumen by Sertoli cells rather than into blood, when compared to boys. It is also possible that men have lower circulating levels of AMH due to a reduction of AMH production by mature Sertoli cells in men, compared to immature forms in boys (183). The persistence of adult levels of AMH in men in the studied cohorts, even from the youngest participant (19 years) suggest that none of the men had testes that were similar to pre-pubescent boys.

A minority of men (4%) in the Otago cohort had AMH levels above the upper limit of reference range. It had been postulated that older testes undergo de-differentiation of their Sertoli cells in some of the seminiferous tubules, leading to elevations of plasma AMH levels (47, 48), such as that found in boys. *A priori*, the InhB levels should be low, which was not observed in any of these men, thereby indicating that immature Sertoli cells is not a prominent mechanism to account for the high AMH levels.

4A.4.2 AMH profiles are different between younger and older men

AMH levels in younger men had characteristics that were on average different to older men. AMH levels were not significantly different between different age groups under 50 years [Fig. 4.2B], a finding that was consistent with other cross-sectional studies (41, 64). The levels of InhB likewise were stable for men under the age of 50 years, with a range that is similar to a clinically defined normal range obtained from a clinical laboratory in another centre in New Zealand (Canterbury Health Laboratories, Christchurch). This suggests that Sertoli cell function and number is maintained in men younger than 50 years (176-178, 184).

Men over the age of 70 years had AMH and InhB levels that were more variable than younger men, with average values being less than half that of younger men. One possible explanation for this observation is an age-related attrition of Sertoli cells, which is supported by histological studies (185, 186). Consistent with this, a minority of older men in both Otago and Vascular cohort (3% and 2% respectively) had undetectable levels of both AMH and InhB, indicating that these older men have complete cessation of Sertoli output (187), analogous to all postmenopausal women with regression of their ovarian follicular cells. The loss of one Sertoli hormone in older men was strongly associated with loss of the second Sertoli hormone suggesting that diminishment of Sertoli hormones is occurring at a cellular level. The relationship between loss of Sertoli cells and Leydig cell hormones is examined in Chapter 5.

This variability of AMH is unlikely to be attributable to the presence of diurnal fluctuations, or sporadic influences like testosterone, as demonstrated in the preceding Chapter.

4A.4.3 Relationship between the Sertoli hormones

AMH level is dissociated with InhB level (R^2=0.22 Pearson linear regression), particularly amongst all younger men and some older men. This indicates that

both Sertoli hormones are possibly independently regulated, or have different functions. Both hormones have been individually used in literature as markers of Sertoli number or function (188-191), and this finding suggests that studies of Sertoli function requires the examination of both AMH and InhB.

AMH however is not totally independent to InhB due to its partial correlation, which is statistically significant, with this linkage possibly due to both hormones being derived from Sertoli cells. The linkage is strongest in some older men, particularly at low levels of both hormones, putatively due to Sertoli cell loss. If either hormones have functional significance in older age, their levels should be maintained by remaining Sertoli cells through increased hormonal output (176), which was not evident in these older men. This suggests that the loss of Sertoli cells in these men significantly overwhelm any homeostatic mechanisms to maintain AMH and InhB levels.

A greater proportion of men in the LiLACS group (12%) had undetectable levels of AMH than both Otago and Vascular cohorts. These men in the LiLACS group were older than the other two cohorts [Table 4.2, Fig. 4.4], and majority had concurrent low InhB levels, raising the possibility that the degree of Sertoli cell attrition maybe related to advancing age. Interestingly the remaining two men with undetectable AMH level had low normal levels of InhB, raising the possibility of dysregulation in Sertoli cellular function. The LiLACS group had a significantly higher proportion of Māori men compared to the other two cohorts, but the ratio of LiLACS participants with low AMH levels were similar between Māori and non-Māori suggesting that Māori ethnicity was not a major determinant of AMH levels in older men.

The Vascular cohort, which was free from cardiovascular disease, had higher levels of AMH and tended to have a higher AMH to InhB ratio. The most obvious difference between the cohorts is the proven absence of cardiovascular disease in the Vascular cohort. The importance of this is unknown, although it is consistent with recent observations linking AMH to atherosclerosis in rhesus monkeys (192) and to the diameter of abdominal aorta in healthy men (193).

InhB levels were similar between the older men in the three cohorts despite the cardiovascular differences between the Vascular cohort and the other two groups.
There was a difference in ethnicity, as Māori men on average had statistically significant lower levels of InhB but not AMH than non-Māori men, with the biological relevance of this currently unknown.

4A.4.4 Strengths and Limitations of study

The comparison of two additional cohorts to the Otago cohort, allows the first exploration of the Sertoli cell hormonal profiles in the context of biology or pathology. Each cohort therefore provides different contextual information. The Otago cohort is an unselected cohort, and examines the profiles of Sertoli cell hormones across the adult male lifespan. This is the most representative of community men. The Vascular cohort at the other end of the spectrum is a highly selected subset of men in the same population that is devoid of any cardiovascular disease and atypically healthy. Comparison between the two cohorts in the Otago region provides insight that allows generation of hypothesis to elucidate function of AMH in men. Similarly, the inclusion of the LiLACS cohort with ethnic and geographical disparity to the two Otago cohort enriches the study and allows generation of further hypotheses to guide further specific studies on AMH.

The cross-sectional nature of this study means that secular changes such as intergenerational differences of AMH levels cannot be excluded, and better answered in a longitudinal study that is outside of the scope of this thesis.

Whilst AMH has moment-to-moment stability in blood, the stability across days, weeks or months have not been determined for AMH or InhB. This includes more chronic sporadic influences such as the effect of seasonality on the variability between men. The three cohorts were individually recruited and sampled for their blood throughout the year. The age-matched average AMH levels were not

different between the Otago and the LiLACS cohort, suggesting that these sporadic variabilities such as seasonality are not a major determinant in the circulating level of AMH. This is in keeping with a limited study in InhB in young men that showed InhB to be invariant to the effect of seasonality (194). To definitively assess for seasonality, a longitudinal study assessing AMH levels taken at several times during the year would be informative.

The age-related changes for AMH are unknown and presumed to be a continuous variable. The correlation of age-related changes to traits would require a study of much larger sample size. This is beyond the scope of this exploratory study that was set out to compare changes between the young and the older males. The age cut-off of 50 years is arbitrary, in order to facilitate statistical analysis between the two age groups.

Another limitation of the study is the sampling of blood at different times of day, which may limit the implication of the findings in a clinical context, where blood is commonly sampled in the morning. AMH levels, however, have been shown to invariant to the time of the day [Chapter 3].

4A.5. Conclusion

AMH persists across the male lifespan and into the very elderly, with variability between men. This variability increases with age. AMH exhibits partial independence to InhB, suggesting that the regulation of these two Sertoli hormones is largely independent from each other. This mirrors the distinct daily profiles of these hormones as demonstrated in Chapter 3. The relationship between AMH and InhB however have some partial linkage, with the strength of this coupling increasing at lower levels of both hormones, indicating loss of Sertoli function and/or number for some older men. The following Chapter will examine whether this loss of Sertoli function influences the level of Leydig cell output, and vice versa. The role of AMH is unknown, with putative roles in the cardiovascular system, but is outside the scope of this thesis.

Chapter 4B AMH levels in postmenopausal women

4B.2 Methods

21 women aged 65 years and older were recruited from the community in Dunedin. Flyers were advertised around the local hospital and University. The majority of the women were Caucasian. Participants were excluded from the study if they had a history of oophorectomy, ovarian malignancy, on oestrogen hormone replacement therapy, radiation therapy to pelvis, or chemotherapy.

A comparison group consisting of 9 healthy young women with regular menstrual cycles was recruited via flyers advertised in similar venues to the postmenopausal women. Women were excluded from the study if they had a history of polycystic ovarian syndrome, on hormonal treatment including hormonal contraception, and chronic medical illnesses.

A single non-fasting venous sample of 4mL was collected from the antecubital fossa vein from each participant, and assayed for the AMH value.

This study was approved by the University of Otago Human Ethics committee.

4B.3 Results

Postmenopausal women, with mean age of 76.9 years (range 65-108 years) have either undetectable (18 out of 21 women) serum AMH levels, or their levels just above the limit of detection [Fig. 4.6, 1.13-2.76 pM]. There were no discriminating features between the subgroup with detectable AMH and those without, in relation to age at sampling, age at menopause, or ovarian pathology. 9 healthy ovulating young women's serum AMH levels were measured in the same assay as the postmenopausal women to ensure that the ELISA kit utilised were able to detect normal age-based values of AMH. The mean age for the younger women was 22.4 years, and their mean AMH level was 36.4 pM (range 3-82 pM), consistent with normal values for younger women (72).

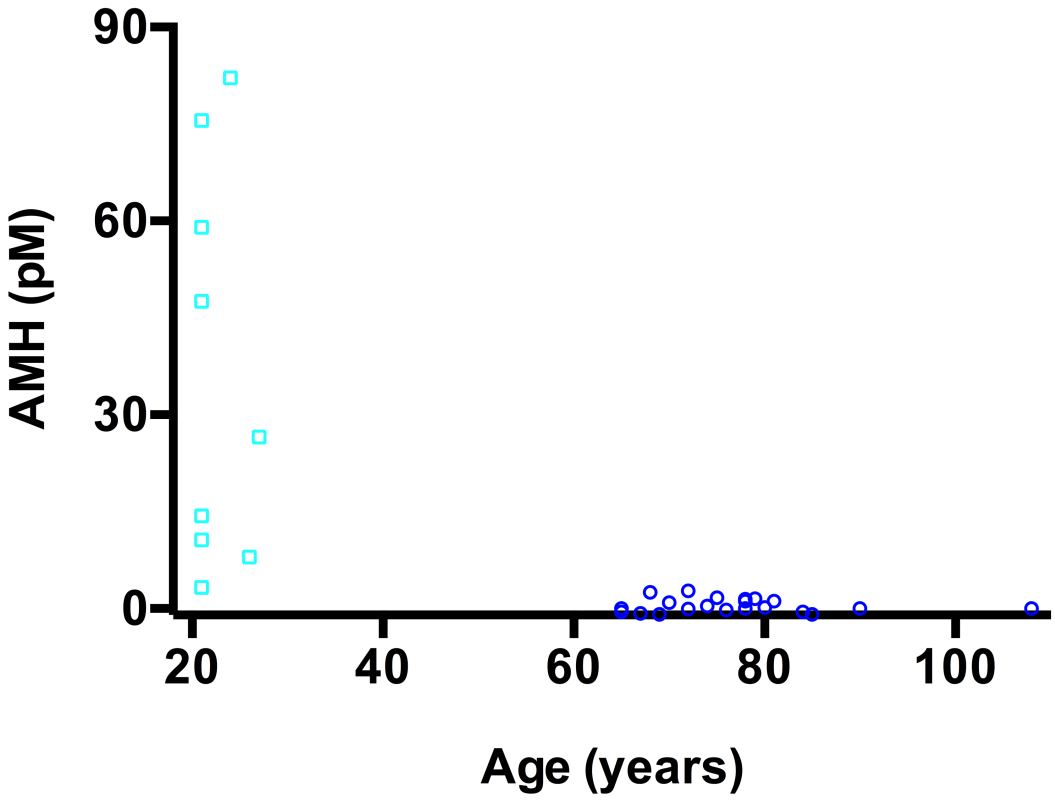

Figure 4.6: Serum AMH levels in women.

Each dark blue circle represents an older postmenopausal women and showed undetectable AMH levels, in contrast to the younger healthy women represented by light blue squares.

4B.4 Discussion

This data demonstrates that the loss of AMH in women prior to menopause persists into senescence, in contrast to squirrel monkeys (179). Therefore, subsequent Chapters in this thesis have not studied women, as this falls outside the scope of the thesis, which has a focus on ageing, and AMH. However, this data may be informative regarding the role of AMH in older males, with this Chapter showing the sexual dimorphism of AMH with ageing. Some older men have undetectable AMH levels like all postmenopausal women, and these older men may putatively have ageing traits more commonly seen in older women.

This information may also be useful for the detection of granulosa cell tumours, a form of ovarian malignancy typically first presenting in the peri-menopausal period. Long-term follow-up is recommended after treatment, as the tumours frequently reoccur, up to 37 years after resection (195, 196). Granulosa cell tumours can be associated with elevation of oestradiol and inhibin, which are used as biomarkers for recurrence. However, oestradiol is falsely normal in up to 30% of granulosa cell tumours (197). Similarly, inhibin lacks specificity, as it can also be elevated in epithelial ovarian tumours (198, 199). The absence of AMH in postmenopausal women makes this hormone an ideal biomarker for detection of granulosa cell tumours following resection amongst women in this age group.

The average level of serum AMH in elderly women was not significantly different from the lowest detection limit of the ELISA. However, a minority of women had values marginally higher than the detection limit. This may be explained by statistical variation around the zero value of the ELISA or alternatively maybe secondary to a low level of AMH production in a minority of elderly women. In support of the latter argument, trace level of AMH can be immunoprecipitated from the serum of some elderly women (M. Pankhurst, personal communication). This suggests that a minority of elderly women have small numbers of residual granulosa cells in their ovaries, which may modify their background risk of developing late-onset granulosa cell tumours.

Chapter 5:

Profile of circulating testicular hormones in younger and older men

5.1 Introduction

The circulating levels of AMH and InhB differ between men, with age-related decline [Chapter 4]. The regulation of Sertoli cell is complex, and involves the expression of androgen receptors during puberty [Chapter 1.4.3]. Testosterone is postulated to mediate the decrease of AMH level during puberty [Chapter 1.4.3]. The influence of testosterone on Sertoli hormonal output is unknown, but if the effect of testosterone persists during adulthood, then linkage should occur between the Sertoli and Leydig hormones. Conversely, non-physiological studies have shown that AMH can impact on the production of testosterone in Leydig cells [Chapter 1.4.2]. The relationship between INSL3 and Sertoli hormones are currently unknown. This Chapter will examine the relationship between testicular hormones in blood of a cohort of men, in a normal physiological model (young men). This will be compared to a cohort of older men to examine if the age-related decline seen in Sertoli hormones is associated with any changes in Leydig hormones.

5.1.1 Profiles of Leydig hormones across lifespan

Circulating testosterone level in man has been extensively studied across the lifespan. Similar to the observation for Sertoli hormones, there is an age-related decline, although occurring earlier than the Sertoli hormones (200-204). Consequently, up to 50% of men in the ninth decade of life have circulating testosterone level below the normative range for young men (205). The significance of the age-related decline is widely debated. Testosterone has been used as a biomarker for the expression of the male phenotype in men. For younger men, low circulating testosterone level is commonly associated with symptoms resulting in deficiency of this male phenotype, such as loss of muscle mass and strength, reduction in bone density, and diminished libido and sexual function (172). Clinical thresholds have been defined for diagnosing testosterone deficiency, termed hypogonadism (172). Exogenous testosterone replacement consistently results in ameliorating the symptoms, highlighting the dominant role for testosterone in younger men to maintain the male phenotype (206, 207).

What is less clear is whether the maintenance of the sexual dimorphism in older men is vital for a male's longevity. There is emerging interest in the role of circulating testosterone to aspects of physical function in the older male, ranging from muscle strength and function, to cardiovascular disease and mortality. This will be reviewed in Chapter 7.1.2, but testosterone replacement has not been universally successful in reversing any health decline associated with low testosterone in the older male (208-211). Consequently, having circulating testosterone level that is below the range of younger men may confer survival benefits, with this being putatively suggested in an observational study of castrated men (212).

There is an emerging concept of hypogonadism in the older men, called late onset hypogonadism. There is a disconcordance between the high prevalence of low blood testosterone level in man (up to 50%), and the prevalence of men with

classical clinical features, being estimated conservatively at 2% of older men (213).

It is also unknown whether this age-related decline in testosterone is specific to the hormone, or the loss of the Leydig cell, with the latter being proposed as the more likely mechanism (214-217). INSL3 has been proposed to be a more precise biomarker for Leydig cell function (33, 218) [Chapter 1.4.1] than testosterone. If the decline in testosterone level is predominantly due to a loss of Leydig cell, then a strong association should exist between testosterone level and INSL3 level in blood, particularly in older men but not in younger men. There has only been one cross-sectional study of INSL3 in an Australian cohort that showed age-related decline in men (134). However, this study has an age range spanning from 35 to 80 years old in a single cohort, and is therefore not informative about elderly men.

Levels of INSL3 have been associated with testicular pathology in an inconsistent pattern. Men with low levels of gonadotropins leading low stimulation of testicular function exhibit circulating INSL3 levels that are below the normal range for healthy men (29-31). This is not consistently due to reduced Leydig cell number, as there is a considerable overlap in ranges between men with very low testicular volumes and those without (31). The findings are concordant with studies of Klinefelter's Syndrome where men have primary testicular pathology, but a wide range of INSL3 levels that overlap with levels of men deemed as normal control (29, 30, 219). These men also have disconcordance with their testosterone values. Not all testicular pathologies are associated with low INSL3 however; as men with infertility due to impaired spermatogenesis have levels of INSL3 that are similar to fertile men (29, 30). This may indicate that INSL3 reflects different aspects of testicular function, and therefore the levels maybe independent to the other three testicular hormones.

5.1.2 Objectives of study

This Chapter will profile the circulating levels of four testicular hormones in a cohort of young men, to assess for any interaction between the Sertoli and Leydig hormones. An examination of the four testicular hormones in blood of older men will then be conducted, and assess if the age-related decline is linked between the hormones. This will inform whether hypogonadism is invariantly due to a loss of total testicular output, or if there are different forms of testicular hormonal decline that can lead to better prediction of clinical hypogonadism in the older male.

5.2 Methods

5.2.1 Participants

The cohort studied was derived from the Otago cohort, as described in Chapter 4A.2.1A. Younger men are defined as men younger than 50 years, whilst older men are aged 70 years and older.

5.2.2 Hormonal assays

Four testicular hormones were assayed and examined as described in Chapters 2.1.4 and 2.1.5, and [Table 2.1].

5.2.3 Statistical analyses

The hormonal profiles across the lifespan were represented by the geometric mean and its confidence intervals, grouped by decades in younger men, and every 5 years for older men. The ranges of hormonal values are different for each decade, with this potentially being skewed if the data is represented by the arithmetic mean. The geometric mean (nth root of the product of n values) was chosen instead to provide a more accurate representation of the data. Statistical differences between the groups were tested using one-way ANOVA and Scheffe test post-hoc applied to examine for the differences between sub-groups.

Relationships between individual hormones were examined by Pearson's linear regression [Chapter 2.2.].

The Sertoli cell and Leydig cell each produce two hormones. The relative output for each cell type was examined using the log of the ratio of the two hormones, which were first normalised to the mean of younger men, aged under 50 years [Chapter 2.2.].

Table 5.1 Characteristics of four testicular hormones in younger and older men of the Otago cohort. 111 men were aged under 50 years (mean 36.2 years), whilst 98 men were aged 70 years and older (mean 78.2 years). Two men under 50 years and one man over 70 years lacked samples for testosterone.

Hormone	Testosterone (nM) 6.0-42		INSL3 (ng/mL) 0.4-4.8		AMH (pM) 13.5-121		InhB (pg/mL) 62-311	
Age group	<50 years	≥70 years	<50 years	≥70 years	<50 years	≥70 years	<50 years	≥70 years
Arithmetic Mean (sd)	17.5 (8.7)	11.8 (5.4)	1.8 (1.1)	1.0 (1.0)	44.9 (25.3)	27.4 (21.8)	175 (63)	103 (57)
Median (range)	16.0 (4.6-43)	10.4 (2-27)	1.4 (0.3-5.5)	0.8 (0-5.2)	38.4 (10.2-147.2)	22.9 (0-103.5)	175 (32-364)	103 (0-260)
% older men below reference range	11		18		27		22	

5.3 Results

5.3.1 AMH levels post field notice

The AMH levels in the Dunedin cohort were re-measured post-field notice [Chapter 2.1.5A]. The pattern of age-related changes in AMH levels [Fig. 5.1] was similar to that described in Chapter 4. The relationship between AMH and InhB was also preserved with the post-field notice data, with there being no statistically difference in the AMH versus InhB regression lines calculated from the pre- and post-field AMH data [Fig. 5.2].

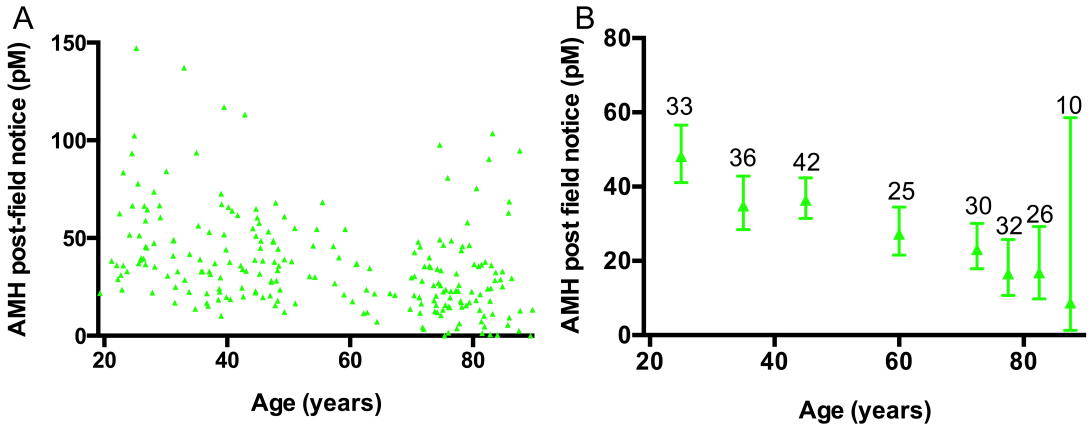

Figure 5.1: Levels of serum AMH post-field notice in Otago cohort.

A. Serum AMH was re-assayed post-field notice [Chapter 2.1.5] with each green triangle representing a man's value. The distribution is similar to that seen pre-field notice [Fig. 4.2A and B]. B. illustrates the geometric mean and 95% confidence interval of AMH in the same cohort.

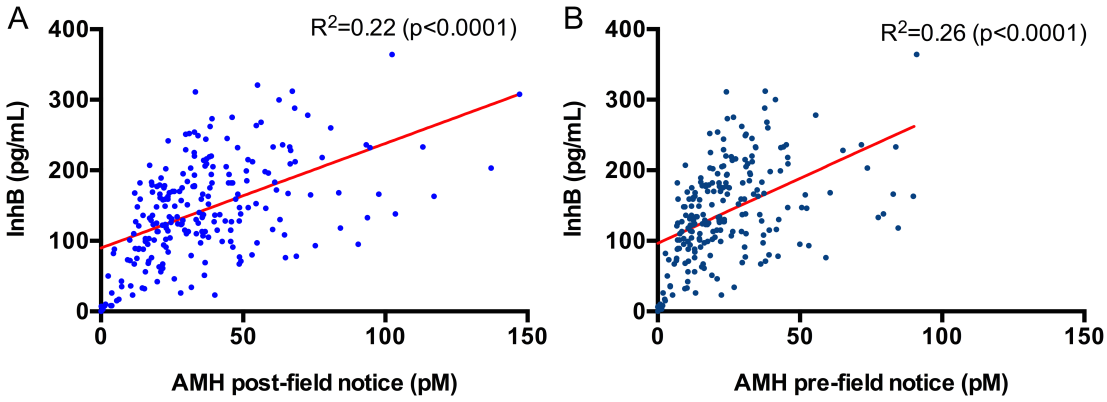

Figure 5.2: The relationship between AMH values post-field notice to InhB is maintained.

A. compared to pre-field notice (B, reproduced from Fig. 4.3B for comparison).

5.3.2 Description of Leydig hormones across lifespan

5.3.2A Testosterone

Testosterone is the most extensively studied of the four testicular hormones in the literature [Chapter 5.1.1]. The men in the cohort demonstrated variability in their testosterone level [Fig. 5.3A and B], with a distribution that was positively skewed in men younger than 50 years (SK test 1.1, $p<0.0001$) and men older than 70 years (SK test 0.50, $p=0.044$). Across the entire cohort, the average testosterone level declined from the fourth decade of life (post-hoc Scheffe test $p=0.0002$), with this being statistically significant [Fig. 5.3B, $p<0.0001$ ANOVA]. This age-related decline in testosterone occurred at an earlier age than AMH or InhB, with both occurring from the 6th decade of life.

5.3.2B INSL3

INSL3 was similarly highly variable between men [Fig. 5.3C], with a distribution that was more positively skewed than testosterone in both younger and older men (SK test 1.2, $p<0.0001$ and 2.3, $p<0.0001$ respectively). The geometric mean for INSL3 remained constant until after 70 years of age, when there was a progressive decline in the average INSL3 levels with advancing age [Fig. 5.3D, $p<0.0001$ ANOVA, post-hoc Scheffe test $p=0.016$], and concurrent increase in variance of INSL3 levels between men. These findings were in concordance with the cross-sectional patterns of the Sertoli hormones.

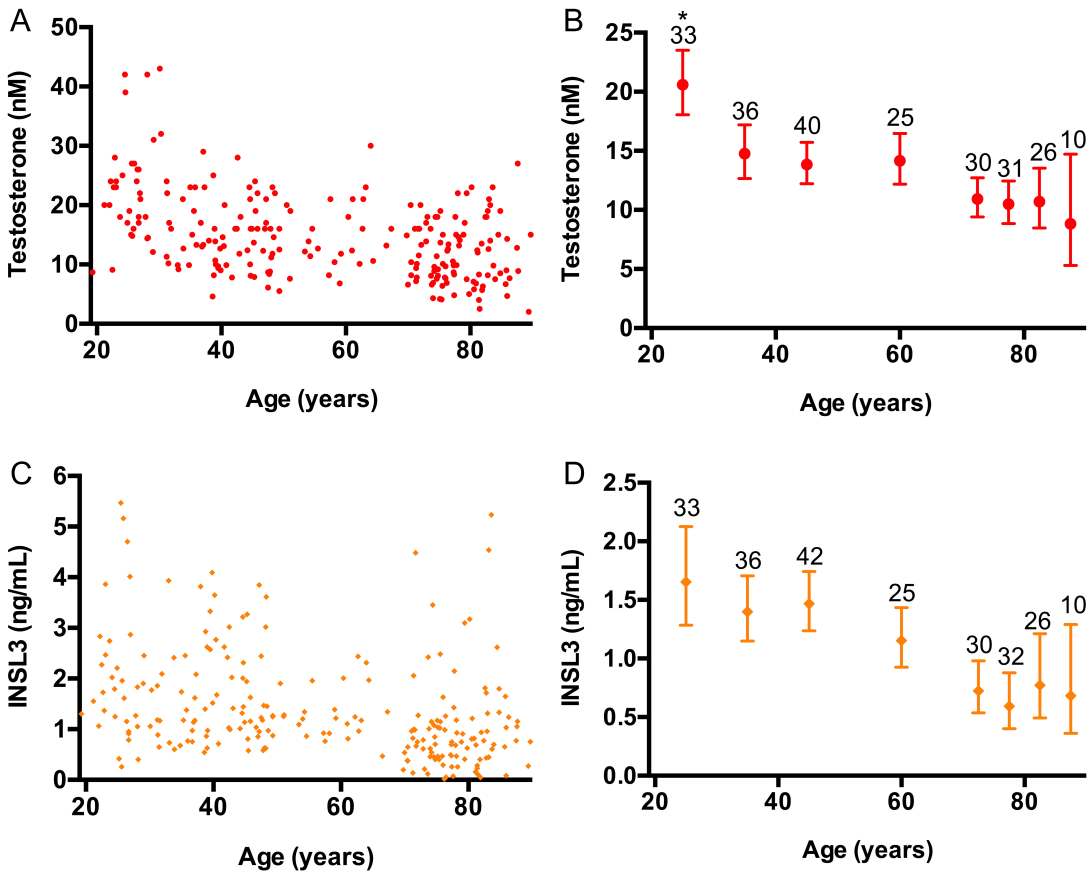

Figure 5.3: The levels of serum Leydig hormones in 233 community dwelling men (Otago cohort) from the cross-sectional study are illustrated across the lifespan of adult men.

A: each red circle represented an individual's testosterone value. B: the geometric mean and 95% confidence interval for testosterone is indicated for various age groups, with the number above the age group indicating sample size. There is a progressive decline in the geometric mean of testosterone from 30 years onwards compared to men in their 20s, which was statistically significant on ANOVA Scheffe post-hoc test (*). C: each orange circle represented an individual's INSL3 value. B: the geometric mean and 95% confidence interval for INSL3 is indicated for various age groups, with the number above the age group indicating sample size. The geometric mean level of INSL3 in men over 80 years is approximately 45% of mean levels found in men under the age of 30.

5.3.3 Testicular endocrine profiles in younger men

5.3.3A Interactions between individual hormones

The variability in individual hormonal levels between younger men prompted the need to examine whether the four testicular hormones are independent of each other. AMH and InhB are largely independent to each other, with 84% dissociation, as shown in Chapter 4 [Fig. 5.4A, R^2=0.16, p<0.0001]. Testosterone and INSL3 showed near complete dissociation to each other despite both hormones originating from Leydig cells [Fig. 5.4B, R^2=0.00, p=0.63]. This phenomenon was also observed between testosterone and the Sertoli hormones AMH and InhB individually [Fig. 5.4C and D, R^2=0.00, p=0.48 to AMH, and R^2=0.00, p=0.50 to InhB]. There was a very weak positive trend for INSL3 correlating to AMH [Fig. 5.4E, R^2=0.06, p=0.010 univariate linear regression] that became statistically insignificant after correction for multiple testing (p=0.06, Bonferroni correction). INSL3 similarly had a non-significant weak positive trend to InhB [Fig. 5.4F, R^2=0.03, p=0.09].

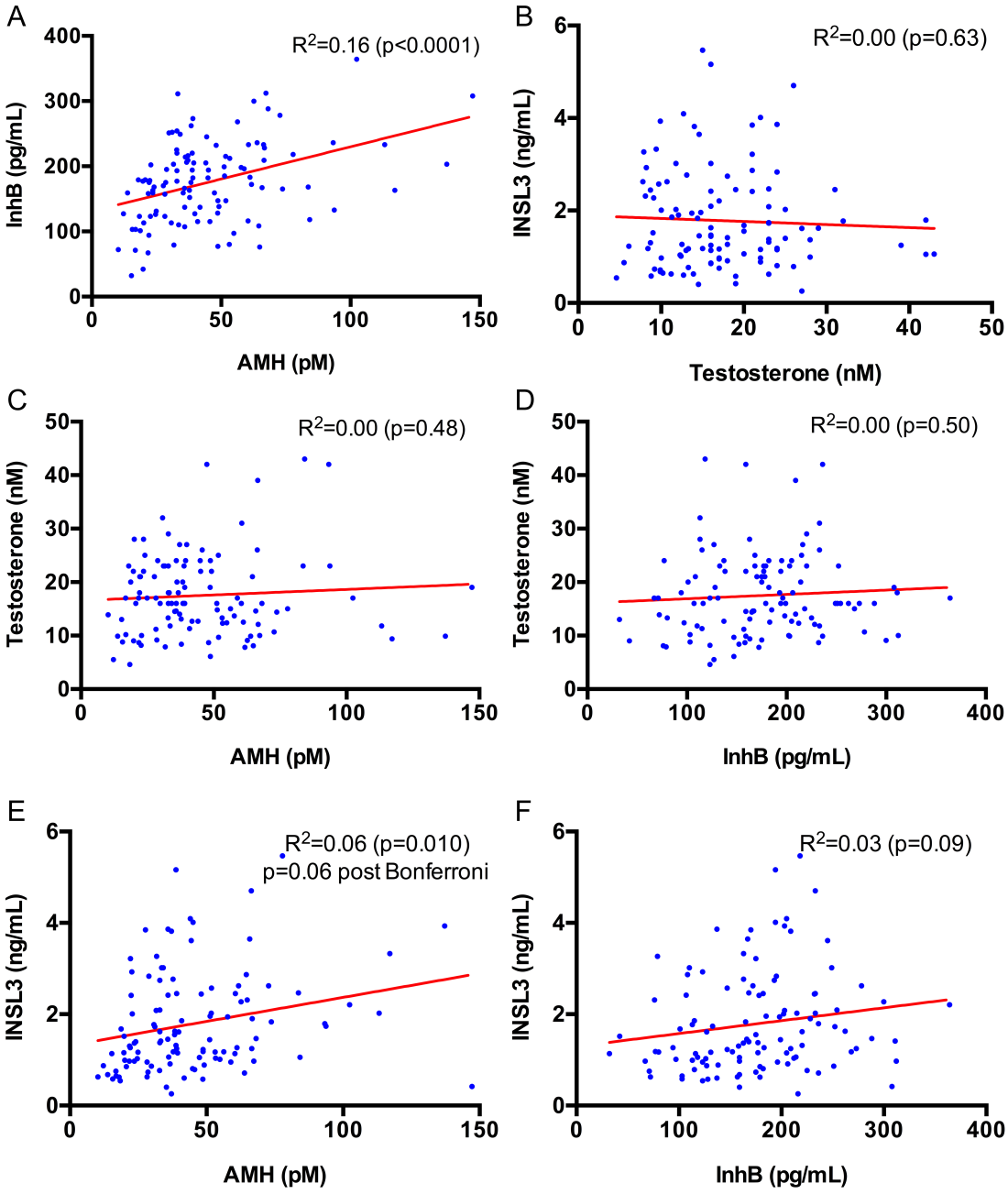

Figure 5.4: The relationship between the different testicular hormones AMH, InhB, testosterone and INSL3 are examined against each other using Pearson linear regression, in the younger men's subgroup of the Otago cohort (19 – 49.9 years).

Each circle represented a participant's hormonal value. The hormones are largely independent to each other. The minor association between AMH and INSL3 was not statistical significance following Bonferroni correction for multiple testing.

5.3.3B Interaction of individual hormones to the relative output of testicular cells

Both AMH and InhB are Sertoli cell products, and similarly testosterone and INSL3 are derived from Leydig cells. The relative output of each cell type can be determined by the logarithm of their ratio, and tested against the individual hormone of the other cell type to determine for any linkages between the two cell types. The relative output of Leydig hormones was independent to the level of the Sertoli hormone AMH (R^2=0.03, p=0.09) and to InhB (R^2=0.00, p=0.57), with no discernable patterns amongst scatter plots graphs [Fig. 5.5A and B]. Similarly, both INSL3 and testosterone had no effect on the relative output of Sertoli cells [Fig. 5.5C, R^2=0.00, p=0.72 for testosterone, and Fig. 5.5D, R^2=0.02, p=0.11 for INSL3].

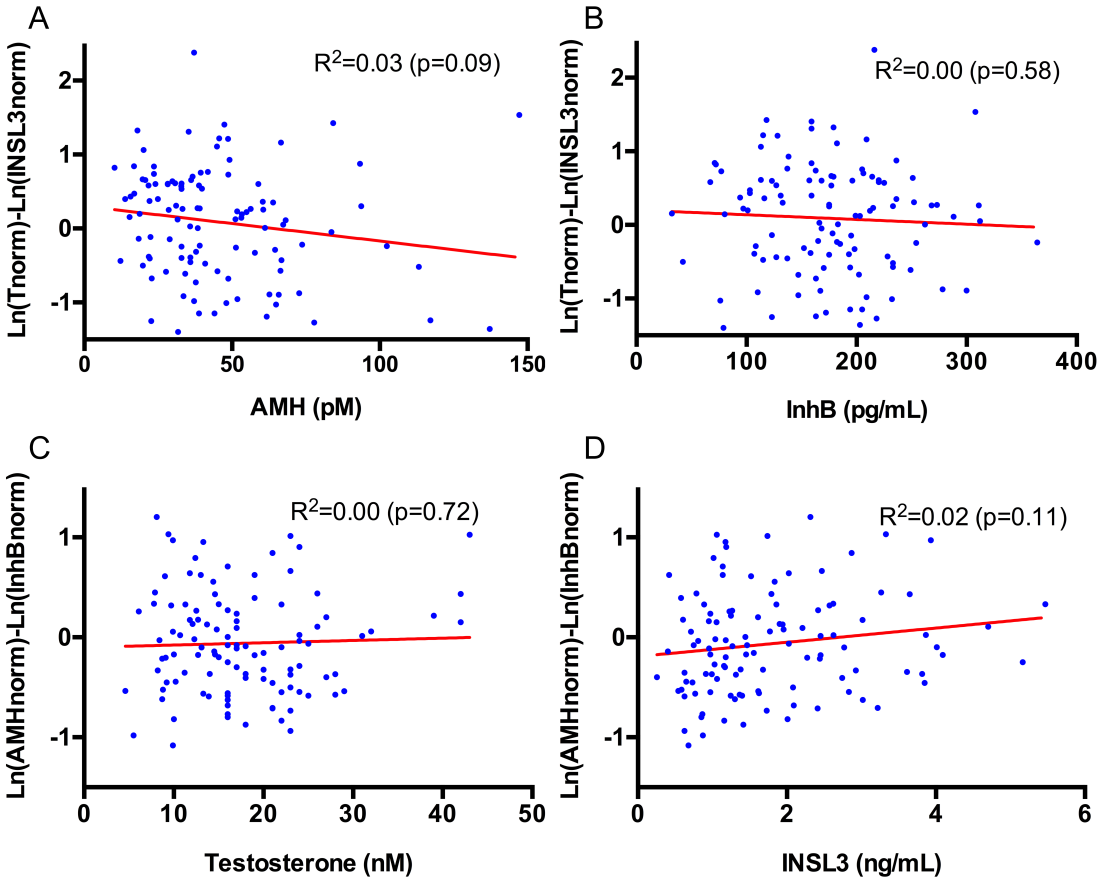

Figure 5.5: Interaction of individual hormones to relative output of testicular cells in younger men.

The relative hormonal output of Leydig cells in younger men is depicted against AMH in A. and InhB in B. Similarly, the relative hormonal output of Sertoli cells is illustrated against testosterone in C. and INSL3 in D. Each circle represented an individual from the younger men's cohort.

5.3.4 Testicular endocrine profiles in older men

5.3.4A Interaction between individual hormones

The independence in the individual testicular hormones was then examined for the cohort of men over 70 years, a group with variable health status and function.

AMH and InhB remained largely independent to each other [Fig. 5.6A, R^2=0.22, p<0.0001]. The weak partial linkage observed was not statistically different to that found in younger men (p=0.21 for differences in the linear regression lines).

There was an emergence of a weak positive correlation between testosterone and the other three hormones with diminishing order of association [Fig. 5.6B, R^2=0.16, p=0.0003 to INSL3; Fig. 5.6C R^2=0.10, p=0.010 to InhB; and Fig. 5.6D R^2=0.06, p=0.09 after correction with Bonferroni for multiple testing]. Given the partial linkage between AMH and InhB, as well as between testosterone and the individual Sertoli hormones, a multiple regression was tested with all three hormonal variables. This showed that the weak relationship between testosterone and InhB remained statistically significant after taking AMH into account (multiple linear regression coefficient 0.24, p=0.022).

INSL3 in older men displayed near total independence to the Sertoli hormones [Fig. 5.6E and F, R^2=0.00, p=1.0 to AMH, and R^2=0.01, p=1.0 to InhB, after Bonferroni adjustment].

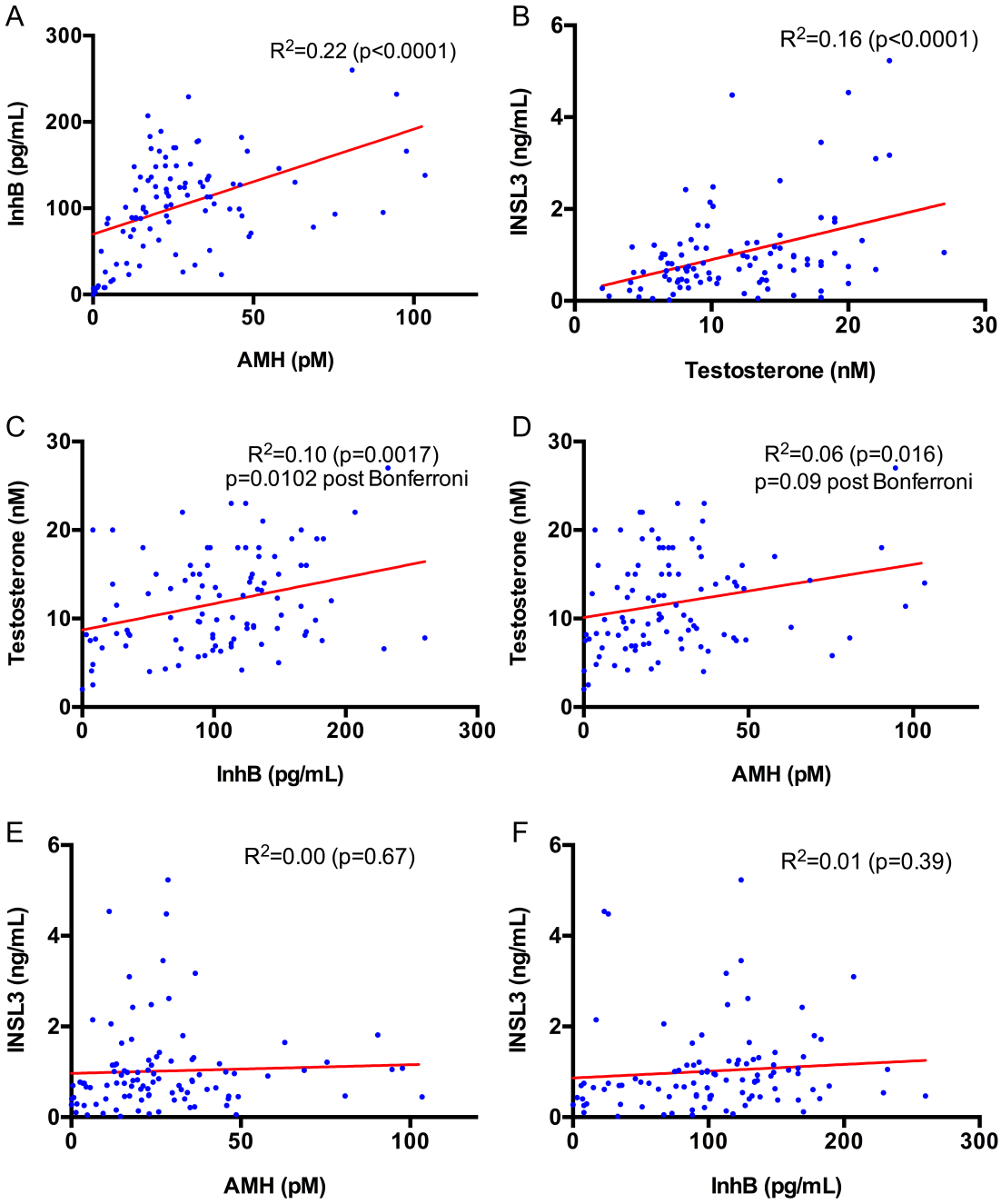

Figure 5.6: The relationship between the different testicular hormones AMH, InhB, testosterone and INSL3 are examined against each other using Pearson linear regression, in the older men's subgroup of the Otago cohort (70 - 90 years).

Each circle represented a participant's hormonal value. There is an emergence of a minor association between testosterone and INSL3, InhB and AMH in decreasing order, with the association to AMH being not significant after correction for multiple testing with Bonferroni.

5.3.4B Interaction of individual hormones to the relative output of testicular cells

The relative output of both Sertoli and Leydig cells were again represented using the logarithm of their hormonal ratio, normalised to their respective mean from the younger men's cohort. If both hormones were declining at a similar rate, their logarithmic ratio would center on zero. The relative output of Leydig hormones in older men however was below zero, indicating that there was greater decline in INSL3 than testosterone in older men [Fig. 5.7A and B]. Both AMH and InhB remained independent to the relative output of the Leydig cells (linear regression R^2=0.00, p=0.94 to AMH, and R^2=0.00, p=0.55 to InhB). Similarly, both testosterone and INSL3 had no effect on the relative output of Sertoli hormones [Fig. 5.7C, R^2=0.0, p=0.52 to testosterone, Fig. 5.7D, R^2=0.0, p=0.50 to INSL3].

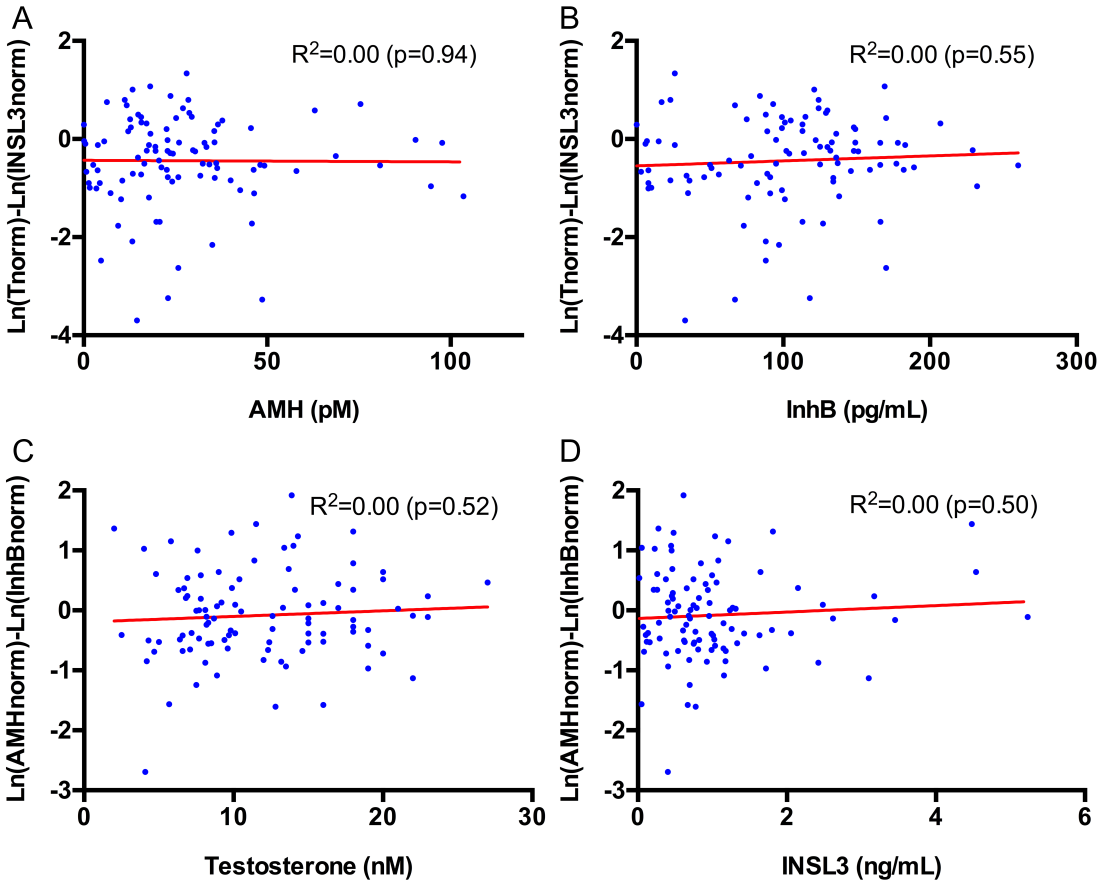

Figure 5.7: Interaction of individual hormones to relative output of testicular cells in older men.

The relative hormonal output of Leydig cells is depicted against AMH in A. and InhB in B. Similarly, the relative hormonal output of Sertoli cells is illustrated against testosterone in C. and INSL3 in D. Each circle represented an individual from the older men's cohort. The individual hormones did not affect the relative output of the cell, which varied between men.

5.3.5 Relative output of the testes

The individual testicular hormonal profiles and its interactions to each other were largely independent in older and younger men, but with heterogeneity. The entire testicular output was then examined in older and younger men to ascertain if patterns emerge across the entire testicular output.

Older men had high variability in their total testicular output. However, a small subgroup of older men had low levels of AMH, InhB and INSL3 with variability in their testosterone level [Fig. 5.8A]. A smaller minority of older men had deficiencies in all four testicular hormones. This pattern was observed to a lesser degree in younger men [Fig. 5.8B]. There was also a clear absence of men in both cohorts with elevated levels of all four hormones [Fig. 5.8A and B].

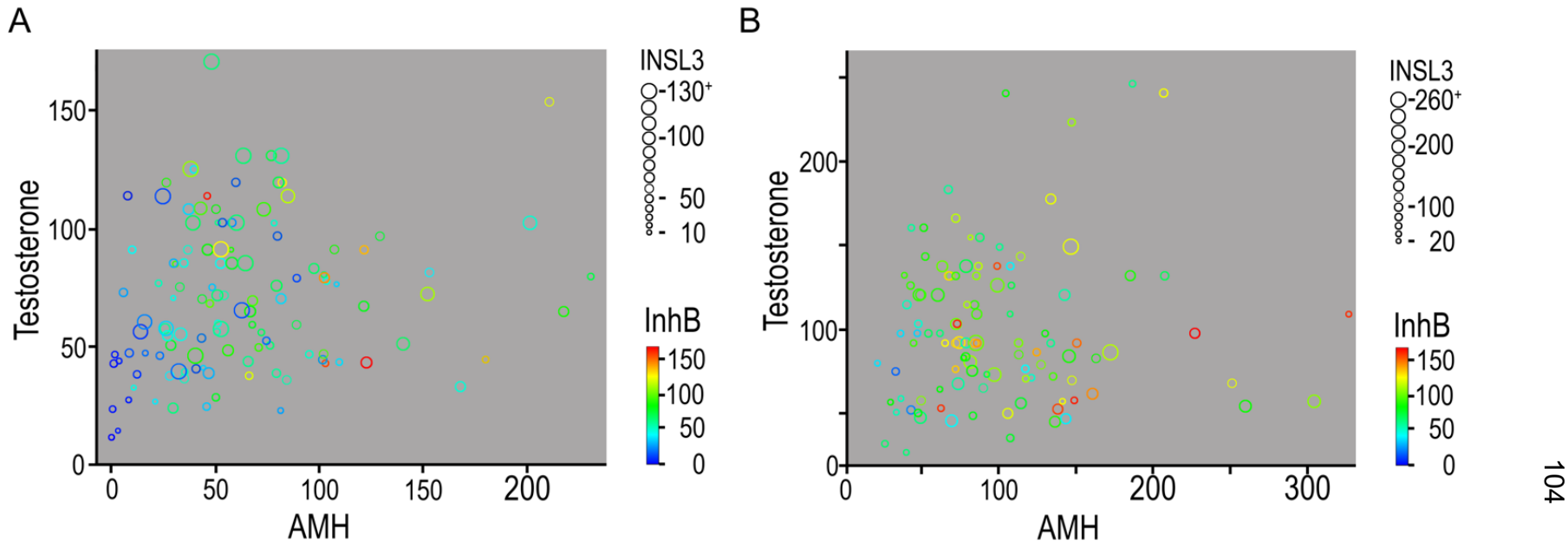
A
Testosterone
AMH
0
50
100
150
200
INSL3
130+
100
50
10
InhB
150
100
50
0
B
Testosterone
AMH
0
100
200
300
INSL3
260+
200
100
20
InhB
150
100
50
0

Figure 5.8: The relative values of all four hormones in the same individual are illustrated for younger men in A. and older men in B.

Each circle represented an individual. The relative values are obtained by normalising the individual hormonal value to the mean value for each respective age group. The relative value for INSL3 is shown by the diameter of the circle, whilst the colour temperature of the circle depicts the relative value for InhB. There is a wide diversity in hormonal profiles in both younger and older men, with a minority in both groups having relatively low levels of all four hormones (lower left of figures, small circles with a blue colour).

5.4 Discussion

5.4.1 The four testicular hormones are independent to each other

This Chapter demonstrated that all four testicular hormones are independent from each other, in both young and older men. This suggests that the regulation and functions of the testicular hormones are largely distinct, and lack commonality to either the cell type or to the testes as a gland. This is particularly evident with INSL3 that showed complete near dissociation to testosterone in younger men. This is consistent with prior studies of Leydig hormones in both healthy men and those with testicular pathologies [Chapter 5.1.2].

The examination of the testes therefore requires the examination of its total output of all four hormones, as Leydig cell hormones and Sertoli cell hormones appear to be distinct from each other. It is likely that all four hormones reflect different aspects of their cellular function, and therefore incongruent with the notion that any single hormone can adequately represent the state of the testes. Consistent with this argument, there has been an approach to measure all four hormones for pathological conditions (55, 219), and this Chapter adds normality data for the interpretation of abnormal states particularly in the older male.

There is an absence of correlation between Leydig hormones and Sertoli hormones, as well as the effect of individual Leydig hormones on relative output of Sertoli cells, and vice versa. This challenges the notion that testosterone regulates Sertoli cell function in the adult [Chapter 5.1.2] which would require a relationship between hormones from the two cell types. This however does not discount the possibility that testosterone may regulate aspects of Sertoli cell physiology, such as its long-term viability, or its effect on germ cells.

5.4.2 Age-related association between hormones are minor

The Sertoli hormones were not completely independent to each other, in both younger and older men, with 16% common variance between the two hormones, with the strength of this relationship being statistically unchanged in older men. This suggests a common factor linking between the two hormones that is invariant to the effect of ageing, with Sertoli cell number being a putative common influence.

In contrast to Sertoli hormones, the Leydig hormones showed age-related coupling evident by a small association in older men but not in younger men. The association appeared to be the strongest when both Leydig hormones are low. This maybe indicative of a critical loss of Leydig cells which crossed the threshold of the testes' ability to maintain differential hormonal levels. This observation requires further validation studies, as this is the first to examine total Leydig cell output in men over 80 years.

5.4.3 Total testicular output profiles are diverse between men

The profiles of the total testicular hormones vary markedly between men across the age spectrum. This is the first study to examine all four hormones in healthy adult men, and indicates that diversity exists within the population of normal men, akin to a biometric of the testes. A minority of older but not younger men exhibited deficiencies in all 4 hormones, and therefore have a global dysfunction of the entire endocrine testes. This is similar to all women following menopause who have near cessation of all their ovarian hormones (220).

The clinical features of men with pan-hypotesticular state are currently unknown. However, these men were heterogeneous in their health status and function with no unifying symptoms. The hypogonadal state of all postmenopausal women may shed light into possible clinical manifestations in men with total testicular failure beyond the loss of testosterone, particularly

with the ageing phenotype. The correlation of testicular hormonal profiles and aspects of phenotype in ageing will be examined in Chapter 7.

Interestingly, there was a clear absence of men both young and old with high levels of all four hormones, despite some men having high levels of single hormones. The significance of this is unclear, but may indicate that possession of a pan-hypertesticular state is not conducive to evolutionary fitness.

5.4.4 Testosterone is preserved in some older men

There was a subgroup of older men with low levels of AMH, InhB and INSL3 that more consistently associated with each other. The testosterone levels in these men were variable. One possibility is that with ageing or in hypogonadism, changes in the protein hormones are occurring prior to changes in the steroid hormones. This is observed in women undergoing the menopausal transition, where the loss of AMH and inhibins preceded the decline in oestrogen by a number of years (74, 221). It is further postulated that gonadal steroidal hormones can be dysregulated when the endocrine function of the gonad is impaired, as seen in menopausal women who exhibits fluctuations in their oestrogen levels (222), and suggested in the testosterone data presented in this Chapter. The hormonal state of the testes has historically been defined by the level of testosterone. The data is incongruent with this premise, as low levels of other three testicular hormones are not consistently associated with low testosterone level, and low testosterone not consistently linked with low levels of other hormones, thereby limiting the predictive value of low testosterone as a strong biomarker for global hypogonadism.

5.4.5 Age-related decline in Leydig hormones

INSL3 exhibited age related decline with high variability between men. This was consistent with the only other study on adult lifespan of INSL3 (134), and suggests that this pattern is independent to the effect of ethnicity and

environmental influences. The wide spread of INSL3 values in the Otago cohort is also observed in the Australian cohort by Anend-Ivell *et al*. This Chapter extended the finding from Anend-Ivell *et al* (134) from men younger than 30, and those over 80 years. Testosterone on average declined at an earlier age than the other three hormones, with this being seen in some epidemiological studies [Chapter 5.1.1].

5.4.6 Limitations of study

This study is a cross-sectional study, and therefore could not exclude secular changes as discussed in Chapter 4.4.4. The sample size was sufficiency to detect statistically significant differences between younger and older men, with subtle age-related effects requiring a much larger sample size typical of epidemiological studies of testosterone. Furthermore, the intention of this study was to examine for any differences between older and younger men, and showed a clear difference between the two groups. This study did not endeavour to profile the entire age range, and therefore lacked a robust enough sample size in the middle-aged group.

Gonadotropins were not measured in this study, as the cross-sectional design was unable to account for the ultradian fluctuations of gonadotropin levels. Consequently, there is lack of consistent statistical correlation between gonadotropins and testicular hormones in epidemiological studies of similar nature (134, 177, 178, 223).

It was observed that for three out of four hormones, the distribution of the hormones was positively skewed rather than normally distributed, in both younger and older men. This requires caution in the utility of a normative reference range, which is usually generated by taking the middle 95th centile values from a reference population group.

5.5 Conclusion

The four testicular hormones AMH, InhB, testosterone and INSL3 have independent circulating profiles in men, both in the young and in the elderly. This results in a total testicular output that is highly variable between men, and suggest that the testes are a complex endocrine organ with multiple hormones that is independently regulated and serves different functions. This is analogous to the anterior pituitary gland that likewise has independent hormonal output.

Older men have on average lower levels of all four testicular hormones, with this being highly variable amongst older men. The independence of all four hormones, and lack of strong association particularly at low levels suggest that hypogonadism can be classified into subclasses with deficiencies in different hormones reflecting different pathologies. Only a minority of older men exhibit a globally low testicular output state, analogous to a pan-hypopituitarism and to all postmenopausal women. The significance of low levels of each hormone and their combinations will be explored in Chapter 7.

Chapter 6:

ProAMH and cleavage to $AMH_{N,C}$

6.1 Introduction

The circulating level of AMH has been described in Chapter 4, and varies between men. The absolute difference in variation however is relatively small in comparison to the decrease in AMH levels typically found in boys prior to puberty (41). The assumption is that the AMH being reported is a single entity. Recently, proAMH has been detected in blood (90), and the current leading commercial ELISA for AMH reports a composite of both proAMH and the cleaved form, $AMH_{N,C}$, which is defined as total AMH by my research group (224)[1]. Consequently, total AMH lacks the ability to discriminate between proAMH and the receptor-competent cleaved form. There has been emerging interest that prohormones, and its cleavage extent, may exhibit functional significance or act as biomarkers [Chapter 1.6.1]. Recently, my laboratory group has developed an ELISA which allows the ability to quantify the amount of proAMH in blood, and by deduction from total AMH, the cleaved $AMH_{N,C}$ can be calculated (129). A calculated ratio of cleavage extent has also been introduced as the API (AMH prohormone index), which describes the proportion of proAMH to total AMH (224). This Chapter will profile the circulating levels of proAMH, $AMH_{N,C}$ and the API in males, with a focus on the comparison between boys and men.

[1] This paper contains data from this Chapter. The citation relates to information that is not part of this thesis.

6.1.1 Cleavage of proAMH occurs in testes

Like all members of the TGFβ superfamily, AMH is initially synthesised as preproAMH. The signal peptide is cleaved intracellularly, resulting in the formation of proAMH (225). Cleavage of proAMH yields a C-terminal component, and a N-terminal component, which remains non-covalently bounded (89). Cleavage of proAMH is thought to occur within the testes, based on a series of experiments from my laboratory group (Pankhurst and McLennan, personal communication). First, recombinant human proAMH protein is not cleaved when incubated in serum. Secondly, intravenous injection of recombinant human proAMH into mice do not result in an accumulation of human recombinant $AMH_{N,C}$. The transgenic mice that do express recombinant human AMH endogenously are able to cleave proAMH to $AMH_{N,C}$. As $AMH_{N,C}$ is AMHR2-competent form of AMH, this raises the possibility that the bioactivity of AMH in blood is regulated by a testicular hormone, and the need to correlate the extent of cleavage of proAMH to the testicular hormones, including AMH itself.

6.1.2 Putative cleavage enzymes of proAMH

Several putative members of proconvertase enzymes are candidates to elicit cleavage of proAMH. This includes the proprotein convertases of subtilisin/kexin-type (PCSK), notably furin (PCSK3), PCSK5 and PCSK6 (226). Plasmin is also able to cleave proAMH resulting in a bioactive form (227). The actions of the PCSKs are broad, as they cleave multiple proproteins (228, 229). The expression and the activation of plasmin and the PCSKs are subject to complex regulation (229, 230), and therefore the extent of AMH cleavage (API) may act as a biomarker to inform about certain states of the body. PCSK4 is expressed by germ cells, and mice with loss of function mutation of PCSK4 are infertile (231). Furin is an important PCSK responsible for the cleavage of multiple members of the TGFβ superfamily (228). Furin can cleave proTGFβ, and the active TGFβ itself can up-regulate furin activity resulting in a positive feedback loop (232). This has been advocated to contribute to the pathogenesis of rheumatoid arthritis (233). Consequently, furin has been identified as

potential therapeutic target (234). Another PCSK enzyme, PCSK9 is now the most topical therapeutic marker in the clinical management of lipid disorders in clinical endocrinology (235), highlighting the emerging importance of consideration of cleavage activities of proproteins.

6.1.3 Objectives of study

The aims of this Chapter is to describe the profiles of circulating proAMH, its cleaved form ($AMH_{N,C}$) and the extent of AMH cleavage (API) in males. The determination of these AMH species and API between boys, young men, and older men may reflect differences in the physiology of these three cohorts. The API will also be correlated to the four testicular hormones, including circulating AMH level itself, to determine if testicular hormones regulate cleavage of proAMH.

6.2 Methods

The data from this Chapter were derived from the analyses of hormonal assays obtained from the participants in the Otago cohort [Chapter 4A.2]. The proAMH assay is described in Chapter 2. A total of 201 men's serum was available to be assayed for their proAMH, leaving 33 men without data for proAMH. 32 out of the 33 men from this cohort were of the younger men's subset, with the remaining individual being older then 70. All of the men with their proAMH not assayed had detectable total AMH levels.

API was calculated for men with detectable levels of both total and proAMH. Men with total AMH levels less than 1.2 pM, and proAMH values less than 1 pM were excluded from the analyses of API, as the variance in their low levels would result in loss of accuracy for the calculation of API.

6.3 Results

6.3.1 Description of the precursor and active AMH forms, cleavage activity across lifespan

6.3.1A Younger men

The level of proAMH was highly variable in men younger than 50 years [Fig. 6.1B and Table 6.1], with a distribution that was positively skewed (SK test 2.6, $p<0.0001$). All younger men had detectable levels of proAMH, with the lowest recorded level being 2.1 pM. The profiles for $AMH_{N,C}$ appeared similar to proAMH, and both mirrored the distribution for total AMH [Fig. 6.1A-C]. The marker of cleavage activity API was distributed across a narrow range (11-25%), and lacked any correlation to age in younger men [Fig. 6.1D, $R^2=0.01$, $p=0.50$ Pearson linear regression].

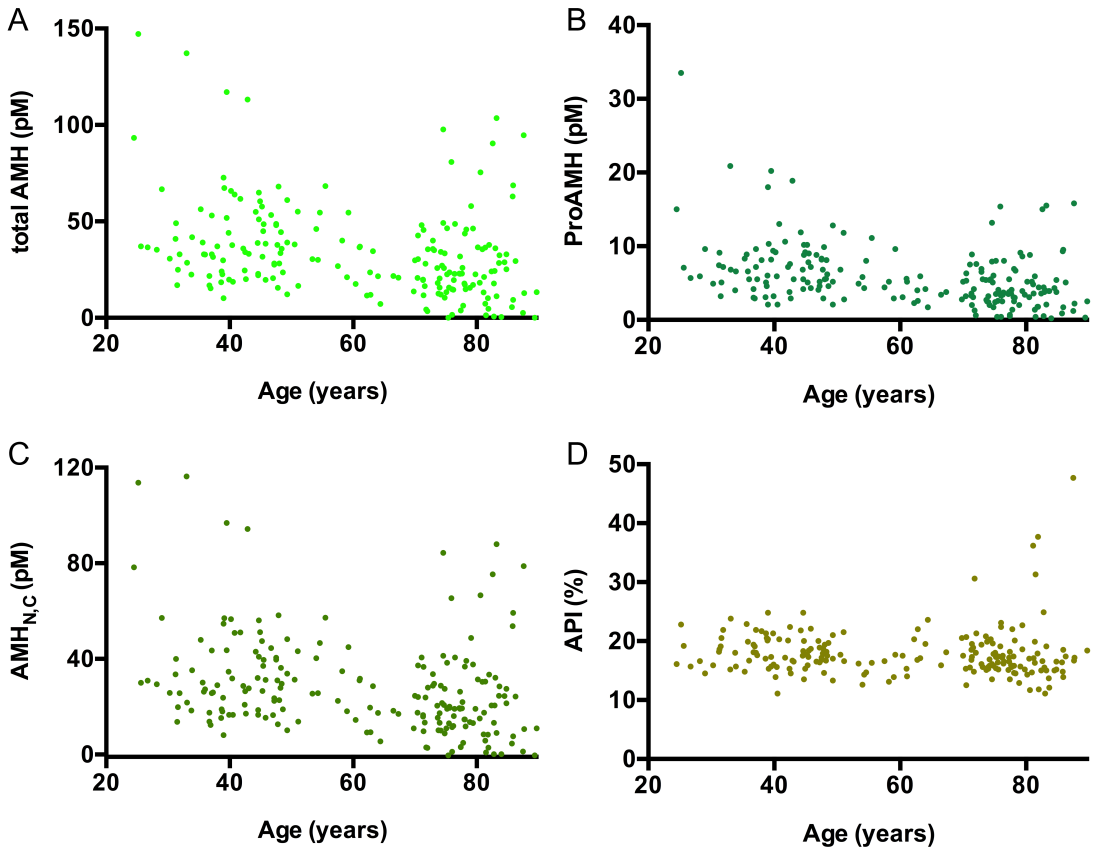

Figure 6.1: The levels of circulating AMH species and API in the Otago cross-sectional cohort from 19 – 90 years.

Each circle represents an individual's value for A. AMH, B. proAMH, C. $AMH_{N,C}$ and D. API.

Table 6.1 Characteristics of circulating AMH species and the API in the Otago cohort of men from 19 – 90 years. 79 men were aged under 50 years (mean age 40.2 years), whilst 98 men were aged at least 70 years (mean age 78.2 years).

	Total AMH (pM)		proAMH (pM)		$AMH_{N,C}$ (pM)		API (%)	
Age group	**<50 years**	**≥70 years**	**<50 years**	**≥70 years**	**<50 years**	**≥70 years**	**<50 years**	**≥70 years**
Arithmetic Mean (sd)	42.8 (26.1)	27.5 (21.8)	7.7 (4.9)	4.5 (3.3)	35.1 (21.5)	22.9 (18.6)	18.2 (2.8)	17.9 (5.4)
Median (range)	36.6 (10.2-147.2)	13.3 (0-103.5)	6.8 (2.1-33.5)	3.8 (0.2-15.8)	29.6 (8.1-116.3)	19.2 (0-88)	18.0 (11.1-24.8)	16.7 (11.1-47.7)
Young vs older men Student's *t* test p value	<0.0001		<0.0001		0.0001		0.64	

6.3.1B Older men

Men aged 70 years and older had significantly lower average values of proAMH compared to younger men [Fig. 6.1B and Table 6.1, 4.5 pM compared to 7.7 pM, $p<0.0001$ Student's *t*- test). In contrast to younger men, in this older men cohort there were 10% of men with undetectable levels of proAMH (1 pM). Older men who lacked proAMH had lower levels of total AMH and $AMH_{N,C}$ when compared to those with detectable levels of AMH ($p<0.0001$ for both with Mann-Whitney U test). However, there was no difference with regards to age between the two groups ($p=0.21$ Mann-Whitney U test). Only half of the men with undetectable proAMH level had concurrent undetectable total AMH, with the remainder being low (range 1.3 - 5.6 pM). The mean levels of $AMH_{N,C}$ were also significantly lower in older men than younger men ($p<0.0001$). The API in older men varied within a narrow range, and on average was no different to those of younger men [Table 6.1, $p=0.64$ student's *t* test]. However, in a minority of men (5%), the API is significantly higher. These elevated API were associated with low total AMH levels [Fig. 6.4A, average total AMH level 5.2 pM).

6.3.2 Relationship between proAMH and $AMH_{N,C}$

ProAMH was strongly associated to total AMH levels (R^2 =0.92, p<0.0001 Pearson linear regression) across the spectrum of total AMH levels in the entire cohort [Fig. 6.2A]. The relationship between proAMH and $AMH_{N,C}$ was strong but not complete (R^2=0.88, p<0.0001), indicating 12% variation between the two AMH species in circulation.

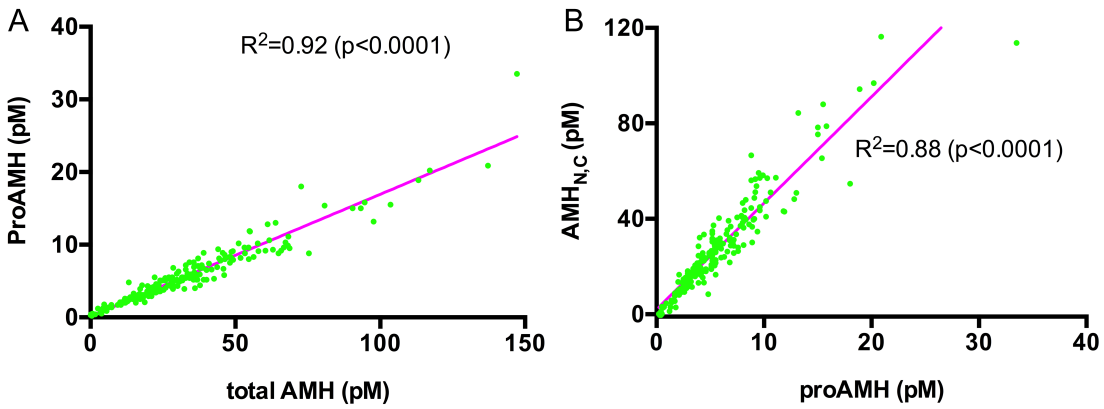

Figure 6.2: The relationship between the circulating AMH species are shown for men in the Otago cohort from 19 – 90 years.

Each circle represents an individual's value. ProAMH is correlated to both total A. AMH and B. $AMH_{N,C}$ with statistical significance.

6.3.3 Relationship between $AMH_{N,C}$ and testicular hormones

The level of $AMH_{N,C}$ was highly correlated to total AMH across a wide range of AMH values, indicating that total AMH is representative of $AMH_{N,C}$ values [Fig. 6.3A]. $AMH_{N,C}$ levels partially correlated with InhB levels [Fig. 6.3B, R^2=0.25, $p<0.0001$], as expected with the partial relationship already known between total AMH and InhB [Chapter 4]. There was near complete dissociation between $AMH_{N,C}$ and the individual Leydig hormones [Fig. 6.3C and D].

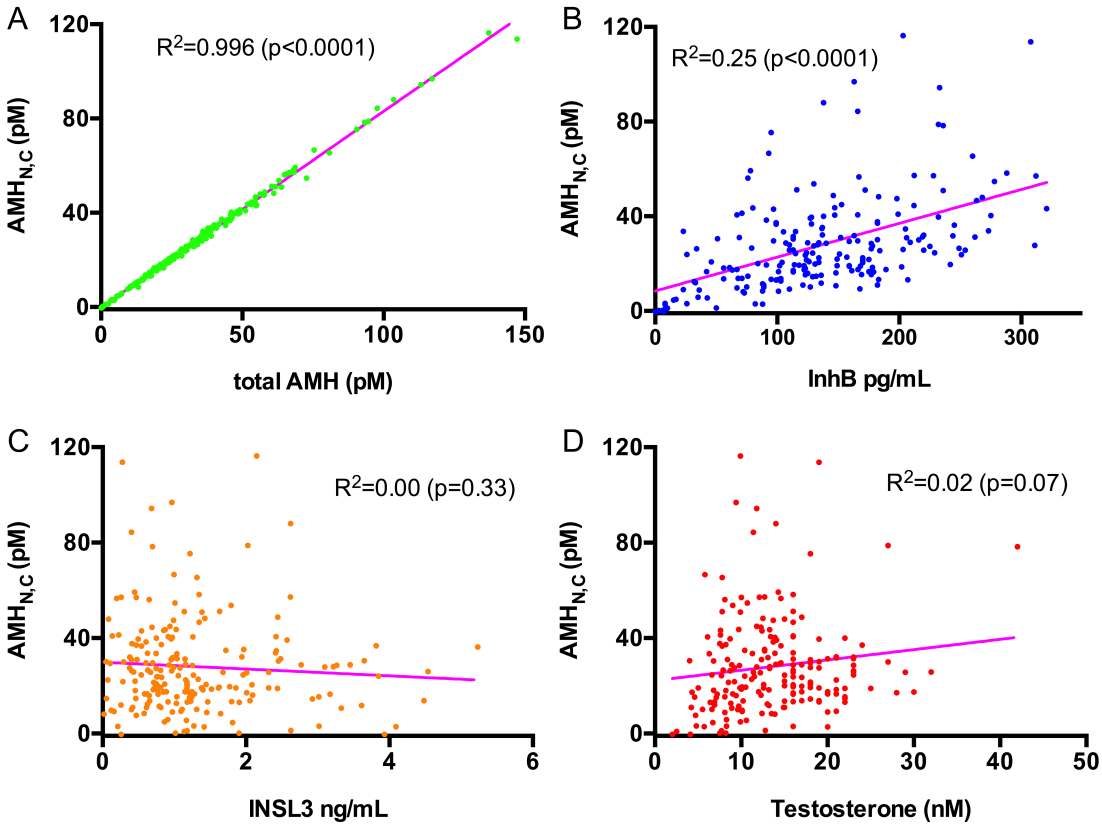

Figure 6.3: The relationship between circulating $AMH_{N,C}$ and individual testicular hormones are depicted for men in the Otago cohort from 19 – 90 years.

Each circle represents an individual's value. $AMH_{N,C}$ correlated to A. total AMH and partially to B. InhB, but was independent to the Leydig hormones C. and D.

6.3.4 Relationship between API and testicular hormones

The extent of cleavage of AMH was independent to the total AMH concentration, in younger men [Fig. 6.4A, R^2=0.01, p=0.32]. The API was also independent to the levels of the other three testicular hormones [Fig. 6.4B-D].

The API was largely invariant to the four testicular hormones in older men, similar to that found in younger men, but with some important differences. There was an emergence of a weak negative relationship between API and total AMH level in older men [Fig. 6.5A, R^2=13%, p=0.0003], which was not observed in younger men. Secondly, there was a minority of men with elevated values of API, all occurring at low values of total AMH. API remained independent to the levels of the other three testicular hormones in older men [Fig. 6.5B-D], mirroring the finding in younger men.

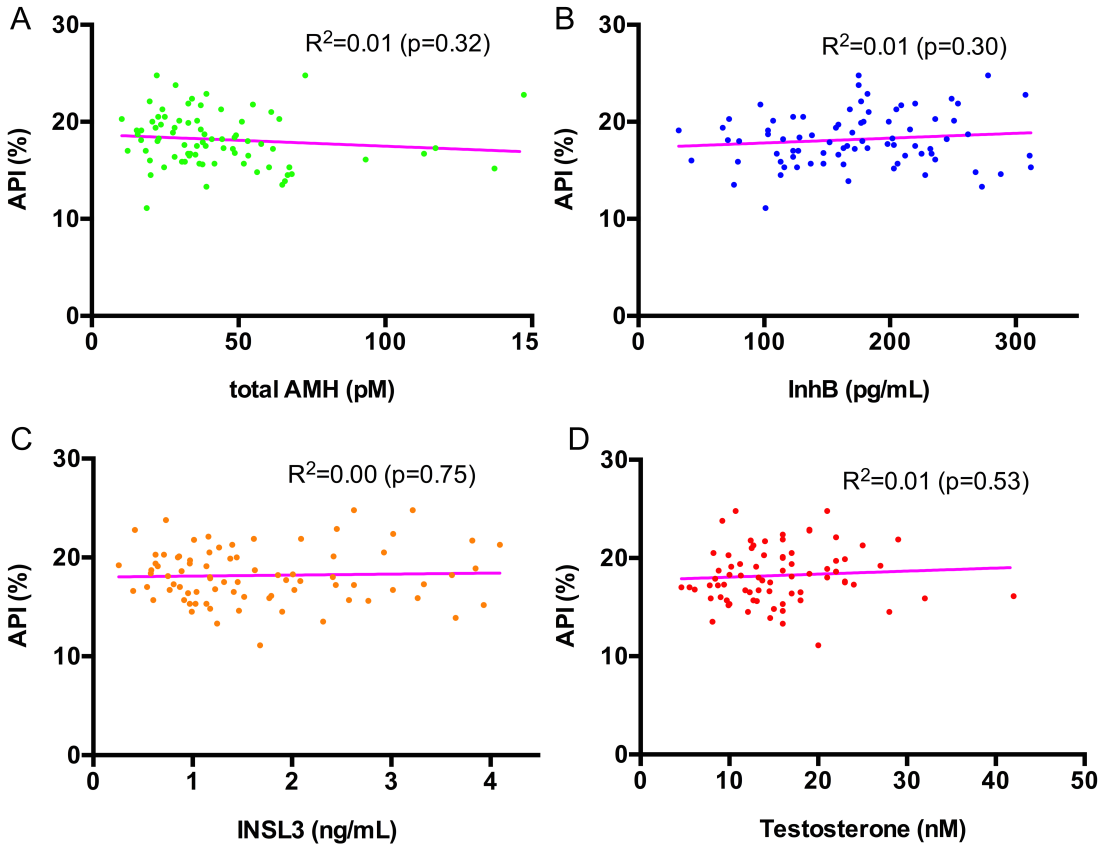

Figure 6.4: The relationship between API and individual circulating testicular hormones are depicted for the younger men in the Otago cohort from 19 – 50 years.

Each circle represents an individual's value. The API was independent to all four testicular hormones in blood of younger men.

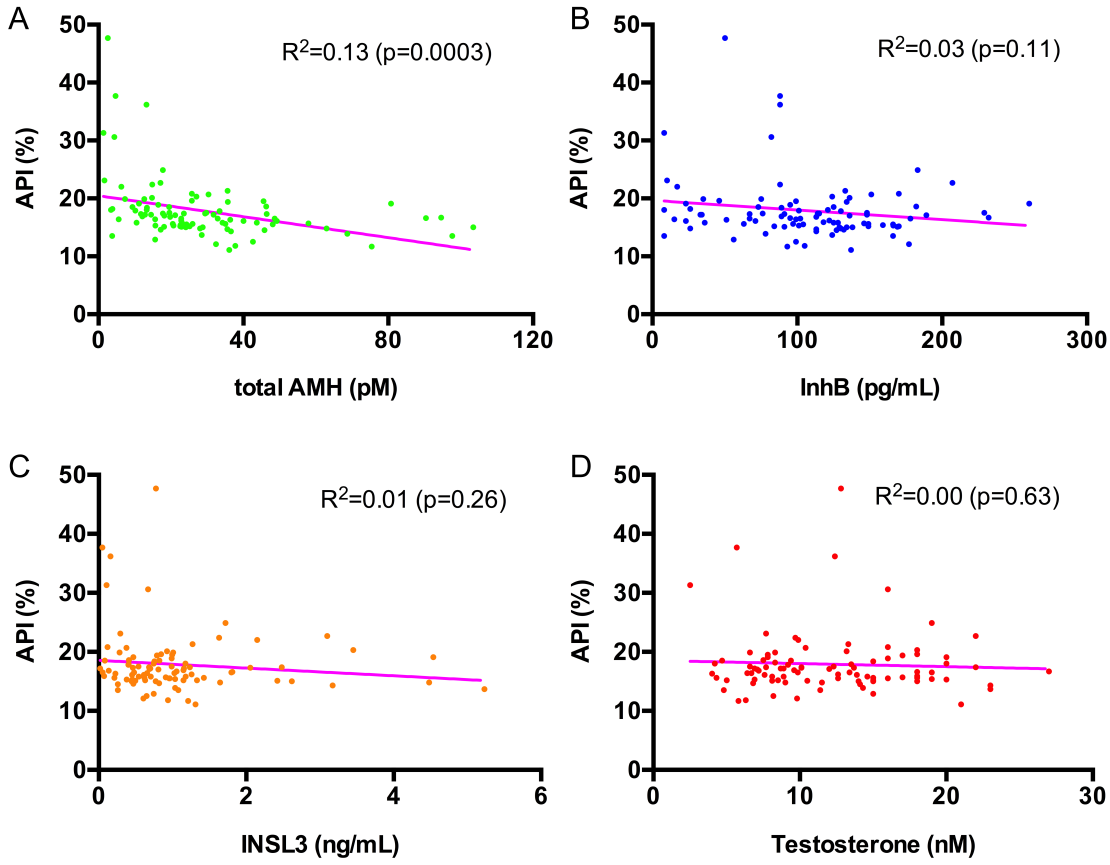

Figure 6.5: The relationship between API and individual circulating testicular hormones in older men 70 years and older in the Otago cohort.

Each circle represents an individual's value. There was a weak relationship to total AMH, but API is independent to InhB, testosterone and INSL3.

6.3.5 API is distinct between boys and men

The level of total AMH is known to be an order of magnitude higher in prepubescent boys compared to men [Chapter 1.5.2 and Fig. 6.6A]. Circulating proAMH levels showed similar difference between boys and men [Fig. 6.6B]. The cleavage activity of AMH for men described above is therefore compared to the data of API of boys that were collected by my laboratory group (with permission to reproduce data here). The mean API for boys was nearly double that of younger men (33.0 compared to 18.2, $p<0.0001$) and to older men (33 compared to 17.9, $p<0.0001$). There was no overlap in the ranges of the API between boys and younger men, as well as the majority of older men [Fig. 6.6C]. The minority of men with higher API levels [Chapter 6.3.4] approached the range in boys.

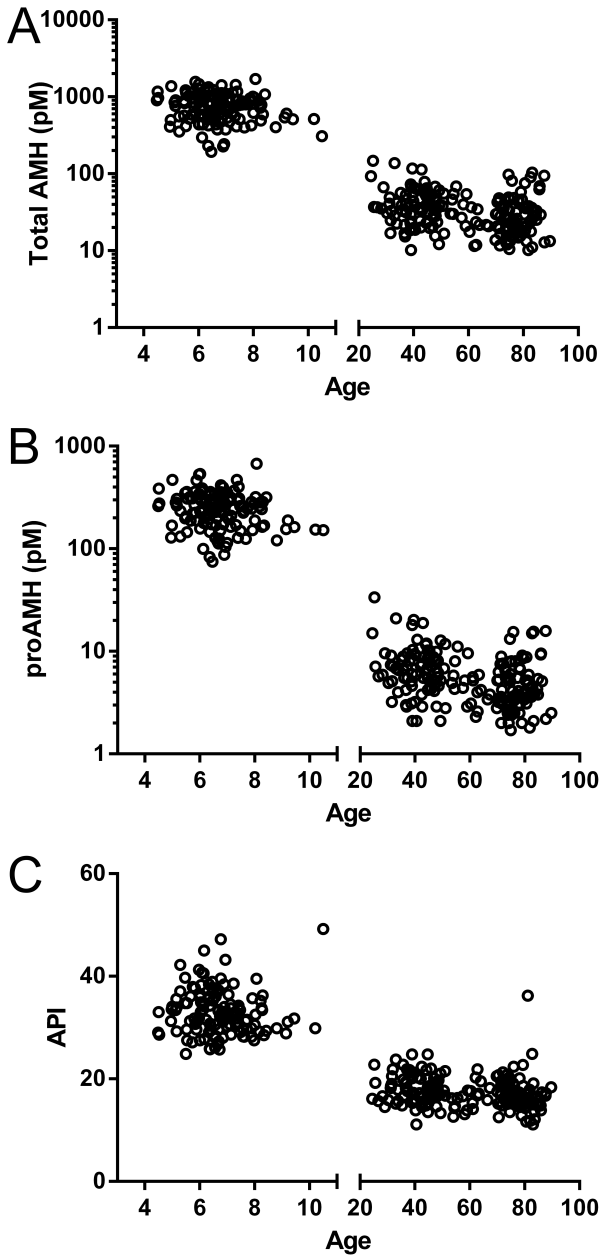

Figure 6.6: Circulating total AMH, proAMH and API are compared between a cohort of prepubertal boys and adult males from the Otago cohort.

Men have approximately 10% of values of total AMH and proAMH compared to boys. The API values for men are distinct to those in boys.

6.4 Discussion

6.4.1 First description of normative range for AMH

This is the first quantitative description of the cleavage of circulating AMH in men. It defines the physiological range for both proAMH and $AMH_{N,C}$ in the serum and provides insight into the regulation of AMH physiology. The normative data provides a reference range for both proAMH and $AMH_{N,C}$, in order to guide observational studies correlating AMH levels and clinical conditions. This can form the first basis for possible utility of AMH as a biomarker for clinical diagnosis. Similarly, the state of the cleavage may be informative about the nature of pathology. Some older men with low AMH levels have higher API values, approaching that typically found in boys. This suggests that for these men with variable health status, the emergence of pathology may lead to less efficient cleavage of proAMH. The clinical significance of disorders of protease activities such as furin and corin in their cleavage of pro-BNP and pro-ANP levels have led to formation of diseases such as salt retaining hypertension and heart failure (236-238). This gives rise to the need to examine API level as a marker of cleavage activities in pathological states and correlating with functional traits.

6.4.2 Regulation of cleavage of AMH

The source of circulating $AMH_{N,C}$ is putatively derived from intratesticular cleavage of proAMH [Chapter 6.1.1]. This raises the possibility that the cleavage of proAMH in the testes is influenced by one of the classical regulators of the testes (LH, FSH) or by one the testicular hormones. If so, then the API index should associate with the circulating levels of one or more of the testicular hormones. Consequently, the absence of any association between the API and circulating hormone levels in health men suggests that the post-translational regulation of AMH is via some other regulatory influence. This argument presumes that intra-person variation in serum levels of testicular hormones is an

accurate biomarker for the level of the hormone adjacent to Sertoli cells. The levels of hormones within the testes are higher than in the circulation (32, 147), with their intratesticular influence modulated by factors such as binding proteins (239). These factors may degrade the relationship between the testicular hormones and the API, but would not be expect to entirely extinguish it, if the testicular hormone(s) were a major regulator. The role of testicular hormones needs to be examined by other experiments, but the current data indicates that other avenues of enquiry should be a higher priority.

Clues regarding regulation of cleavage of AMH maybe derived from the consideration of the fundamental differences between boys and men, as men have more effective cleavage of their AMH than boys, with no overlap between the two life stages. Men but not boys undergo spermatogenesis, and one possibility is that the cleavage of AMH may reflect the state of spermatogenesis in a male. This points to the need to examine conditions in men with impaired spermatogenesis, such as Sertoli cell only syndrome and non-obstructive azoospermia. The testicular hormonal profiles in boys are also distinct to that in men, typically with high total AMH, near undetectable testosterone, low INSL3 and diminished InhB level. This raises the possibility that the intratesticular environment may regulate the cleavage of AMH as evident by the large scale of difference between boys and men, which is not evident when examining smaller magnitude of difference between individuals.

6.4.3 Technical issue

The API is a ratio of values derived from two hormonal assays. Some of the variation between individuals might arise from the assay variations inherent in ELISAs. However, this is not large enough to account for the significant variation of API between boys and men, suggesting real biological association. The coefficient of variation in API is of similar magnitude to that found when examining ratio of the two Sertoli hormones or two Leydig hormones, further indicative that the variation in API between individuals are real.

6.5 Conclusion

Normative data for proAMH, $AMH_{N,C}$ and its cleavage activity has been defined for a group of healthy men, and provides the basis for the next Chapter to examine physical traits in older men with varying clinical conditions, in order to elucidate any functional role for AMH. The regulation of cleavage is not significantly determined by the circulating testicular hormonal levels but showed distinct differences between boys and men that suggest the possibility of API reflecting the state of maturation of the testes. This will be of relevance in the next Chapter to examine whether de-differentiation of the testes is a prominent feature in some older men.

Chapter 7:

AMH and frailty in the older male, in context of other testicular hormones

7.1 Introduction

The objective of this thesis is to advance the premise that circulating AMH has functional significance in men. At the commencement of the PhD, there were no substantial indication as to where AMH might have function in adults, or whether AMH is detectable or even varies between men. AMH may putatively act like a BMP ligand, and therefore AMH might have broad functions [Chapter 1.6.2]. Furthermore, the common intracellular signalling cascade that AMH shares with the BMP subgroup indicates that AMH function may be cryptic, and only observed in conditions where there are multiple dysfunctions in the regulation of similar functions [Chapter 1.4]. This is most likely to occur in an ageing male, particularly in those with frailty. Consequently, this PhD has embarked on a pilot study to determine the feasibility of screening for correlation between circulating AMH levels in a cohort of older men with heterogenous functions. Despite the sequential nature of the thesis, the study performed in this Chapter was conducted concurrently to the determination of AMH and other testicular hormones in blood. Hence there is no a priori hypothesis as to the relationship between AMH and functional traits in the older male. Additionally, an *a priori* power calculation for sample size cannot be

determined, as the coefficient of variation for AMH in the older male was unknown.

7.1.1 The endocrine system in ageing

Many endocrine systems in the older person exhibit age-related changes. Only one system showed an universal cessation of function – that of the female reproductive system with menopause. This has been associated with pathology not otherwise seen in the younger women, and provides clues as to the functional significance of ovarian hormones, including gamete production, the maintenance of the female phenotype, bone metabolism, and cardiovascular disease (240-244). This PhD study on circulating AMH have shown that this cessation of ovarian AMH production is permanent in women [Chapter 4B.3], and that some older men also exhibit complete loss of testicular hormonal output [Chapter 5.3.5].

In most other systems, hormone levels on average are lower in the older person than amongst younger people. The true significance of this is debated. Not all older people manifest symptoms or pathology commonly attributed to the deficiency of a particular hormone, which would be more frequently seen in the younger person. The growth hormone axis is a classical example of this. Growth hormone deficiency can result in short stature in children (245), and in younger adults is associated with osteopenia, poor quality of life and changes to body composition (246-248). Absolute circulating growth hormone level and its surrogate marker insulin-like growth factor-1 (IGF-1) are lower on average in the older person than in the young. There is inconsistent association to symptoms in the older group (249, 250). This age-related change may be an adaptive response for survival, as normalisation of growth hormone level to a younger person's range increases the risk of diseases such as tumour growth and development of diabetes mellitus (251, 252). Consequently, the normative range for growth hormone level is age-adjusted in clinical practice (253).

7.1.2 Late onset hypogonadism

Circulating testosterone level on average declines with ageing [Chapter 5.1.1 and 5.3.2A]. Low levels of testosterone in younger men results in overt pathology of hypogonadism related to the loss of maintenance of the male phenotype. In older men, the association is less clear. This is akin to the situation to growth hormone with ageing. Low testosterone level in the older person have been associated to a number of different conditions, ranging from sarcopenia, frailty, cardiovascular disease, to cognitive impairment and mortality (254-261). Replenishment with exogenous testosterone therapy have resulted in short term improvement such as muscle strength and general vitality. However there are no results of long-term survival benefit, and some evidence of harm (262) including cardiovascular disease (210) and prostate cancer (263). This indicates that low testosterone level may at best be a biomarker for the general loss of homeostasis in the ageing body. The concept is late-onset hypogonadism is being refined, and more strongly associated with sexual function in the older male. Consequently the prevalence of late onset hypogonadism is low at 5% in older men (264). This thesis has expanded on the hypogonadism definition to include the assessment of the other three testicular hormones AMH, InhB and INSL3. This thesis has found that only a minority of men exhibit deficiency in all of their testicular hormones, with each hormone having independent profiles in both older and younger men. This implies that each testicular hormone may serve different functions in men, and some or all of the other testicular hormones may be a better biomarker to inform about the state of the ageing male compared to testosterone. This has not been studied for AMH, InhB and INSL3 at the start of this thesis.

7.1.3 The frailty phenotype

The function of circulating AMH may be cryptic in men, unlike the overt phenotype generated by testosterone. Furthermore, the stable levels of circulating AMH throughout the day is suggestive that AMH may inform about the state of Sertoli cell rather than its acute regulation. This is similar to the posit regarding INSL3 as a Leydig cell marker compared to testosterone. Hence, any

pertubation in AMH function may only be seen following chronic changes, such as in the older male. The characteristic of AMH signalling as a TGFβ superfamily member means that it is possible that AMH may function as part of a complex network with redundancy and interactions [Chapter 1.6.2]. Hence its action may not be immediately obvious unless there is significant dysregulations to this network. A useful working model to assess this is in the frail person.

Frailty is best conceptualised as a reduction in physiological reserve, leading to clinical symptoms due to failure of multiple physiological systems (265). The manifestations of frailty varies according to definition, but common criteria includes pertubations in physical function, gait speed, and cognition (266, 267). Weight loss is also commonly associated with frailty (267, 268). Low testosterone level has been associated with frailty (259, 269). Testosterone replacement do not result in long term amelioration of the frailty phenotype (270). This suggests that testosterone may be a biomarker to other independent factors of frailty, with the presence of other testicular hormones currently being unknown.

The mechanism of frailty or its components are not fully understood. Members of the TGFβ superfamily may play a role in its pathogenesis, with the best documented being myostatin, a member of the BMP subfamily. Myostatin is a known muscle growth regulator (271), and inhibition of myostatin in aged mice resulting in muscular growth (272). However, association studies between muscle strength and circulating myostatin level has been inconsistent (273-275). This does not necessarily prove that myostatin is not a determinant of frailty, as myostatin is subject to complex regulation including cleavage and binding proteins which may impact upon its function in sarcopenia (276).

7.1.4 Objectives of study

The objectives of this Chapter is to explore for any putative association between circulating AMH levels, and its API, to aspects of functional traits. This is a pilot study on a heterogenous group of older men, with some participants displaying features of frailty. The study will be performed in the context of other testicular hormones, to determine if any associations between AMH and functional traits are linked to changes in other testicular hormones. Findings from this study will guide more specific observational studies and potential causality studies.

7.2 Methods

7.2.1 Participants

The participants were derived from the subgroup of Otago cohort comprising of men aged 70 years and older, as described in Chapter 4.2.1A.

7.2.2 Hormonal assays

The circulating hormones assayed were those described in Chapter 5 and 6, including total AMH, proAMH, $AMH_{N,C}$, Inhibin B, testosterone and INSL3. The API was calculated from proAMH and total AMH as per Chapter 6.

7.2.3 Clinical measurements

All tests were conducted in a single session, during the daytime hours between 8am to 3pm. This was performed in an indoor gymnasium facility of the local hospital, which is usually utilised for outpatient physiotherapy rehabilitation.

7.2.3A Anthropometric measurements

Each participant was examined for the following: height (Seca 213 stadiometer), weight (Seca 813 scale), and waist circumference, defined as the transverse circumference midline between the iliac crests and the lowest palpable ribs (277). BMI (Body mass index) was calculated using the following formula: weight (kg) / height $(m)^2$. Waist circumference correlated to weight and BMI with statistical significance ($R=0.87$, $p<0.0001$ for weight, and $R=0.85$, $p<0.0001$ for BMI) and to a lesser degree to height ($R=0.38$, $p<0.0001$) [Table 7.1A].

7.2.3B Physical functional capacity

The physical functional capacity for each participant was examined by the Timed-Up and Go test (278), the Short Physical Performance Battery Test (279), and the 6 minute walk test (280). All three tests have been validated as measures for physical capacity and frailty (278-280). There was partial correlation between all three measures of physical function in this cohort, to high statistical significance [Table 7.1B].

The Timed-Up and Go Test (TUG) measures the time taken to stand from a sitting position, walk to a marker 3 metres away, turn and return to sitting position at origin, all conducted at usual walking speed. The TUG informs about the mobility and balance, and exhibit high interclass correlation (ICC > 0.95) (278).

The Short Physical Performance Battery Test (SPPB) is a composite of three tests, each scored out of 4 to compile a total score of 12. The first test measures the time required to repeatedly stand and sit from a chair, without utilising the arms. This test examines for lower limbs strength and balance. The second test examines for standing balance, ranging from full tandem (one foot directly in front of the other) to a loss of standing balance without aid. The final test records the time required to walk 8 feet (2.44m), which establish the gait speed. Lower SPPB score predicts short term mortality and admission to residential care (279).

The third test of physical function is the 6 minute walk test (6MWT) that measures the distance walked lengthwise throughout the gymnasium, at usual walking speed. The 6 minute walk tests predicts frailty and mortality risk, particularly in patients with cardiopulmonary diseases (280).

7.2.3C Cognitive Test

Similar to the physical testing, a number of cognitive tests were utilised to comprehensively measure cognitive function. The most commonly utilised tool for assessment of cognitive function both clinically and in research is the Mini Mental State Examination (MMSE), a 30 points test that examines a wide range of different components of cognition including orientation, memory recall, attention, calculation, language and visuospatial skills. A diagnostic cut off value of 24 or less predicts dementia, with a specificity of 99%, whilst using a cut off value of 27 or less discriminates between normal cognition and mild cognitive impairment/dementia with specificity of 91% (281).

The Modified Mini Mental State Examination (3MS) is a 100 points test that extends the MMSE, and correlated highly with the MMSE both in this cohort (Table 7.1C, R= 0.84, $p<0.0001$) and in the literature (282). In some clinical centres, 3MS is used in lieu of the MMSE.

Both Trail Making Test A (TMT A) and Trail Making Test B (TMT B) tests for visual scanning, task switching and executive function tests. It involves recording the time required to connect 25 circles placed in a random order on the page, with TMT A examining consecutive numbering, whilst TMT B requires alternating between a number and a letter. Since depression, particularly in the older adult, share similar clinical features with cognitive impairment, a screening validated tool for depression is used in this study. There was partial relationships between Geriatric Depression Scale (GDS) and other tests of cognition [Table 7.1C]. The GDS is a 15 point questionnaire that ask 15 yes/no questions to a range of depressive symptoms, with higher scores indicating greater risk of depression.

7.2.4 Statistical analyses

All univariate analyses between two variables were conducted using Pearson's correlation. Partial correlation was performed when examining for the effect of multiple variables on the relationship between two variables.

Table 7.1: Linear correlation between different tests within same domain, with A. showing anthropometric measurements, B. physical function, and C. cognitive function. Relationships that are statistically significant are presented in blue.

A. anthropometry	Height	Weight	Waist circumference	BMI
Height	-	R=0.61 p<0.0001	R=0.38 p=0.0001	R=0.16 p=0.1
Weight	R=0.61 p<0.0001	-	R=0.87 p<0.0001	R=0.88 p<0.0001
Waist circumference	R=0.38 p=0.0001	R=0.87 p<0.0001	-	R=0.85 p<0.0001
BMI	R=0.16 p=0.1	R=0.88 p<0.0001	R=0.85 p<0.0001	-

B. Physical function	TUG	SPPB	6MWT
TUG	-	R=-0.60 p<0.0001	R=-0.78 p<0.0001
SPPB	R=-0.60 p<0.0001	-	R=0.73 p<0.0001
6MWT	R=-0.78 p<0.0001	R=0.73 p<0.0001	-

C. Cognition	MMSE	3MS	TMT A	TMT B	GDS
MMSE	-	R=0.84 p<0.0001	R=-0.40 p=0.0001	R=-0.66 p<0.0001	R=-0.30 p=0.0034
3MS	R=0.84 p<0.0001	-	R=-0.55 p<0.0001	R=-0.75 p<0.0001	R=-0.34 p=0.0009
TMT A	R=-0.40 p=0.0001	R=-0.55 p<0.0001	-	R=0.45 p<0.0001	R=0.45 p<0.0001
TMT B	R=-0.66 p<0.0001	R=-0.75 p<0.0001	R=0.45 p<0.0001	-	R=0.13 p=0.2
GDS	R=-0.30 p=0.0034	R=-0.34 p=0.0009	R=0.45 p<0.0001	R=0.13 p=0.2	-

7.3 Results

7.3.1 Cohort characteristics

The characteristics of the participants are shown in Table 7.1. The average age for the cohort was 78 years old, with a mean weight of 80 kg, an overweight BMI of 27 kg/m^2, but normal mean waist circumference of 98 cm. The physical performance measures were variable in the description of the cohort, with the TUG being and SPPB being of normal range for the age group, whilst the mean 6MWT was lower than reported average in literature, but with high variance (283, 284). The average cognitive scores were also in the normal range for the age.

Table 7.2: Characteristics of 99 men aged 70 years and older in the Otago cohort

Features	Mean (sd)	Median (range)
Age (years)	78.1 (5.1)	77.4 (66.5-89.7)
Height (m)	1.71 (0.06)	1.71 (1.53-1.86)
Weight (kg)	79.9 (12.6)	77.8 (55.0-123.2)
Waist circumference (cm)	97.8 (9.5)	98 (80-124)
BMI (kg/m^2)	27.3 (3.4)	27.0 (19.3-37.2)
Time Up and Go Test (seconds)	10.7 (7.6)	9 (5-78)
Short Performance Battery Test (0-12)	10.1 (2.4)	11 (0-12)
6 minute walk test (m)	293.7 (76.7)	303 (0-422)
15 point Geriatric Depression Scale (0-15)	1.2 (1.7)	1 (0-10)
Modified Mini-Mental State Examination (0-100)	93 (8)	96 (44-100)
Mini Mental State Examination (0-30)	28 (2)	28 (17-30)
Trail Making Test A (seconds)	46.3 (20.0)	41 (19-127)
Trail Making Test B (seconds)	134.3 (151)	90 (27-960)

7.3.2 AMH correlation to features

7.3.2A Anthropometric markers

The API was inversely associated to a person's weight and BMI [Table 7.3A, R=-0.24, p=0.02 for weight, and R=-0.23, p=0.02 for BMI]. This relationship was maintained after correction for age and waist circumference, as age and waist circumference are independently associated to weight and BMI [Table 7.3B]. Obesity was associated with more efficient cleavage of AMH [Fig. 7.2A and B], whilst men with normal or low BMI exhibited a wide range of API.

The circulating levels of total AMH, proAMH, and $AMH_{N,C}$ however showed no relationship to weight or BMI. Elevated waist circumference is a marker of truncal obesity, and none of the AMH hormones or the API displayed any association to waist circumference. Similarly, there was no relationship to height.

Table 7.3A: The linear univariate correlation R and its statistical significance is described between AMH and anthropometric measurements in 99 men 70 years and older. Bonferroni correction for multiple testing is shown in parentheses. Relationships that are statistically significant are presented in blue.

	Height	Weight	Waist circumference	BMI
Age	R=-0.35, p=0.0004 (p=0.004)	R=-0.38, p=0.0001 (p=0.001)	R=-0.15, p=0.14	R=-0.27, p=0.007 (p=0.07)
Total AMH	R=0.14, p=0.16	R=0.09, p=0.35	R=0.08, p=0.44	R=0.03, p=0.77
proAMH	R=0.16, p=0.11	R=0.05, p=0.59	R=0.03, p=0.76	R=-0.03, p=0.79
$AMH_{N,C}$	R=0.14, p=0.17	R=0.10, p=0.32	R=0.09, p=0.40	R=0.04, p=0.69
API	R=-0.11, p=0.27	R=-0.24, p=0.02 (p=0.22)	R=-0.15, p=0.16	R=-0.23, p=0.02 (p=0.2)

Table 7.3B: Partial correlation of API to weight and BMI, after correction for age.

	Weight	BMI
API	R=-0.22, p=0.03	R=-0.22, p=0.03
Age	R=-0.33, p=0.001	R=-0.20, p=0.05

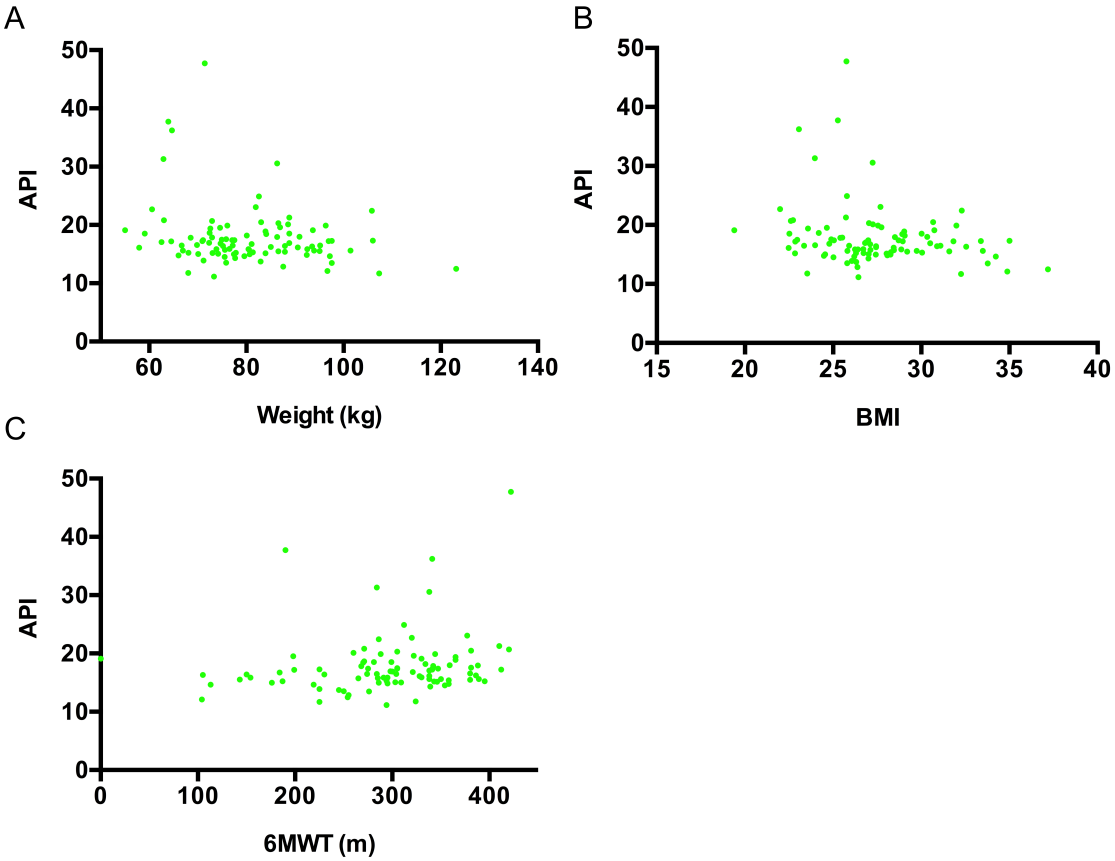

Figure 7.1: Relationship between API and characteristics of men aged 70 years and older in the Otago cohort.

Each dot represents an individual. The API was inversely associated to both A. weight and B. BMI, and as well as to the C. 6 minute walk test.

7.3.2B Physical frailty

Advanced age was associated with physical frailty with high statistical significance (R=-0.31, p=0.002 for SPPB, and R=-0.32, p=0.002 for 6MWT). There is greater cleavage of AMH in participants that are more frail, after correcting for the effect of age [Table 7.4, partial correlation 6MWT to API, R=0.23, p=0.03]. At higher API, there is a absence of men with poor physical function from the 6MWT [Fig. 7.2C]. Circulating levels of AMH hormones were not associated with frailty in both univariate analyses and after correcting for the effect of age.

Table 7.4: The linear univariate correlation R and its statistical significance is described between AMH and physical measurements in 99 men 70 years and older. Bonferroni correction for multiple testing is shown in parentheses. Relationships that are statistically significant are presented in blue.

	TUG	SPPB	6MWT
Age	R=0.12 p=0.24	R=-0.31 p=0.002 (p=0.01)	R=-0.32 p=0.002 (p=0.01)
Total AMH	R=-0.10 p=0.32	R=0.06 p=0.58	R=-0.06 p=0.58
proAMH	R=-0.12 p=0.24	R=0.07 p=0.46	R=-0.00 p=0.96
$AMH_{N,C}$	R=-0.10 p=0.35	R=0.05 p=0.60	R=-0.06 p=0.53
API	R=0.03 p=0.7	R=0.07 p=0.49	R=0.19 p=0.07

7.3.2C Cognitive domains

MMSE is a broad measure of cognition, and widely utilised to assist in diagnosing and monitoring cognitive impairment. MMSE negatively associated with circulating levels of total AMH (R=-0.23), proAMH (R=-0.24) and $AMH_{N,C}$ (R=-0.23) [Table 7.5A, Fig. 7.2]. Cognitive decline is associated to depressive symptoms, to a high level of significance, with both markers being linked to age. A partial correlation was therefore performed between circulating AMH hormones and cognitive function as measured by MMSE, correcting for the effect of age and the GDS score. The partial correlation was strengthened slightly between circulating AMH hormones and MMSE [Table 7.5B]. ProAMH similarly improved its strength of relationship to 3MS, another marker of cognitive function, after correcting for the effect of depressive symptoms [Table 7.5B]. Conversely, there was no relationship between circulating AMH hormones and depressive symptoms in both univariate and partial correlation after correcting for cognitive function and age. API was independent to all tests of cognitive function and depression.

Table 7.5A: The linear univariate correlation R and its statistical significance is described between AMH and cognitive measurements in 99 men 70 years and older. Bonferroni correction for multiple testing is shown in parentheses. Relationships that are statistically significant are presented in blue.

	MMSE	3MS	TMT A	TMT B	GDS
Age	R=-0.28 p=0.007	R=-0.29 p=0.004	R=0.26 p=0.01	R=0.18 p=0.08	R=0.26 p=0.01
Total AMH	R=-0.23 p=0.03	R=-0.17 p=0.11	R=0.09 p=0.37	R=0.13 p=0.21	R=-0.04 p=0.72
proAMH	R=-0.24 p=0.02	R=-0.18 p=0.08	R=0.06 p=0.53	R=0.15 p=0.15	R=-0.06 p=0.56
$AMH_{N,C}$	R=-0.23 p=0.03 (p=0.27)	R=-0.16 p=0.11	R=0.10 p=0.34	R=0.13 p=0.22	R=-0.03 p=0.8
API	R=0.07 p=0.5	R=-0.07 p=0.5	R=0.02 p=0.8	R=0.06 p=0.6	R=-0.04 p=0.7

Table 7.5B: Partial correlation of MMSE and 3MS to AMH markers, corrected for effect of age and GDS score. Relationships that are statistically significant are presented in blue.

	MMSE	**3MS**
Total AMH	R=-0.25 (p=0.02)	
proAMH	R=-0.27 (p=0.009)	R=-0.21 (p=0.049)
$AMH_{N,C}$	R=-0.25 (p=0.02)	
API	R=0.07 (p=0.5)	

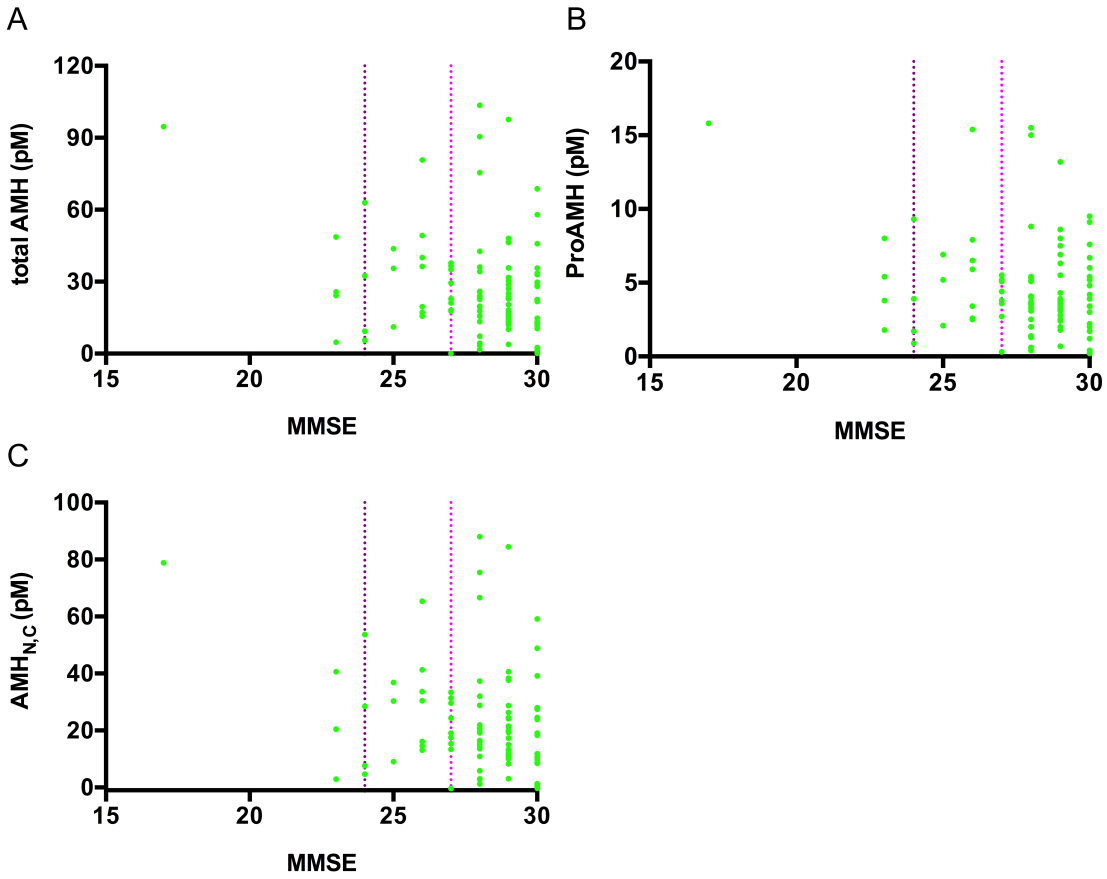

Figure 7.2: Relationship between the various circulating AMH measurements and the mini mental state exam (MMSE) in men aged 70 years and older in the Otago cohort.

Each dot represents an individual. AMH showed negative correlation to MMSE score with statistical significance. A MMSE of 27 or higher (dark purple dashed line) is associated with normal cognition. MMSE score less than 24 (light purple dashed line) is associated with cognitive impairment.

7.3.3 Context of other testicular hormones

Relationships were observed between circulating AMH hormones or the API, to physical and cognitive traits in older men. Post-hoc analyses were then performed to determine if these associations were distinct to AMH, or similar in the context of other testicular hormones.

7.3.3A Anthropometric measurements

No relationship was observed between weight and BMI and the circulating levels of InhB, testosterone and INSL3. Circulating INSL3, in contrast to circulating AMH, associated to a man's height with statistical significance, after correcting for the common effect of age [Fig. 7.3A and Table 7.6, partial correlation R=0.25, p=0.03]. This appeared to be specific to circulating levels of INSL3, as neither the circulating levels of testosterone nor InhB showed any association, in both univariate and multivariate analyses.

7.3.3B Physical frailty

Lower circulating levels of InhB was associated with increasing frailty with statistical significance (linear regression to TUG, R=-0.20, p=0.046, Fig. 7.3B), with the relationship preserved after partial correlation to age [Table 7.7]. This observation was unique to InhB only, and not seen with AMH, testosterone or INSL3. However, the statistical effect appeared to be influenced by a single individual with poor function of TUG and low InhB, with the strength of statistical significance being lost when the data was re-analysed after exclusion of this individual (linear regression p=0.17).

7.3.3C Cognitive function

There were no relationship between circulating levels of InhB, testosterone and INSL3 to measures of cognition and depression, in both univariate and partial correlation studies correcting for age and depression [Table 7.8]. This was in contrast to the statistical significant associations observed between circulating AMH hormones and cognition.

Table 7.6: Univariate linear regression between circulating levels of InhB, testosterone and INSL3 to anthropometric measures in 99 men 70 years and older. Relationships that are statistically significant are presented in blue.

	Height	Weight	Waist circumference	BMI
InhB	R=0.09 P=0.4	R=-0.03 P=0.8	R=-0.06 P=0.6	R=-0.08 P=0.4
Testosterone	R=0.08 P=0.5	R=-0.07 P=0.5	R=-0.05 P=0.6	R=-0.13 P=0.2
INSL3	R=0.29 P=0.05	R=-0.01 P=0.95	R=0.03 P=0.8	R=-0.13 P=0.2

Table 7.7: Univariate linear regression between circulating levels of InhB, testosterone and INSL3 to physical function in 99 men 70 years and older. Relationships that are statistically significant are presented in blue.

	TUG	SPPB	6MWT
InhB	R=-0.21 p=0.046	R=0.19 p=0.06	R=0.05 p=0.6
Testosterone	R=-0.11 p=0.2	R=0.05 p=0.7	R=0.08 p=0.4
INSL3	R=-0.10 p=0.3	R=-0.03 p=0.7	R=-0.14 p=0.2

Table 7.8: Univariate linear regression between circulating levels of InhB, testosterone and INSL3 to cognitive function in 99 men 70 years and older. Relationships that are statistically significant are presented in blue.

	MMSE	3MS	TMT A	TMT B	GDS
InhB	R=-0.14 P=0.2	R=-0.05 P=0.6	R=-0.06 P=0.6	R=0.14 P=0.2	R=-0.6 P=0.5
Testosterone	R=-0.10 P=0.3	R=-0.09 P=0.3	R=-0.11 P=0.3	R=0.13 P=0.2	R=-0.04 P=0.7
INSL3	R=-0.09 P=0.4	R=0.04 P=0.7	R=-0.02 P=0.8	R=-0.02 P=0.9	R=0.2 P=0.1

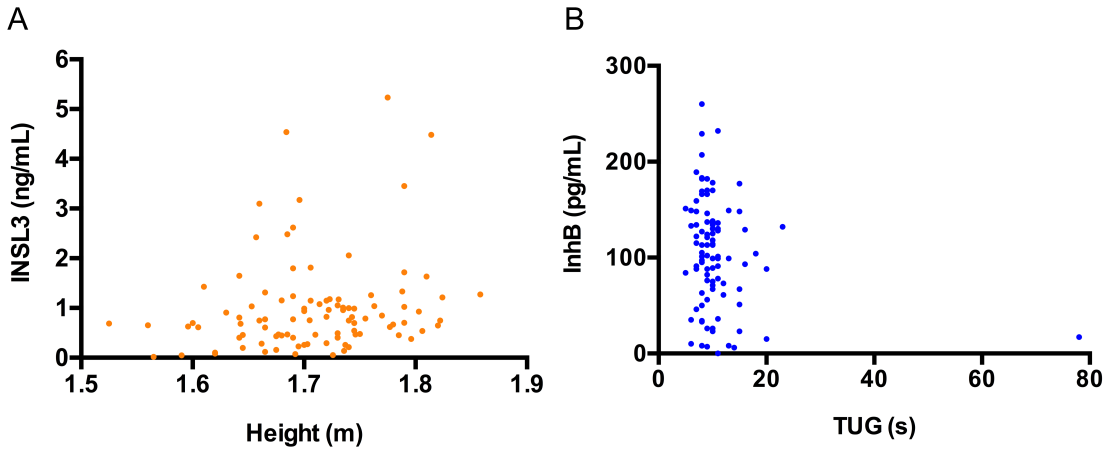

Figure 7.3: Relationship between selected testicular hormones and traits in men aged 70 years and older in the Otago cohort.

Each dot represents an individual. A. Higher levels of circulating INSL3 are associated to taller height. B. Individuals with longer time on Time-Up and Go test have lower InhB levels.

7.4 Discussion

To date there is an absence of knowledge regarding functions of circulating AMH in man. This Chapter described the world's first exploration of circulating levels of AMH and its cleavage extent in a cohort of older men with diverse functional capacity. This pilot study has identified the feasibility of conducting further research to define functions of circulating AMH in specific domains.

7.4.1 Circulating AMH level and cognition

Circulating AMH level inversely correlated to cognitive function in older men. This points towards a possible area for investigation of function of AMH. Any functional significance for AMH in the brain is likely to be broad, as the association is the strongest to MMSE and 3MS, which are broad measures of cognition. Interestingly, the relationship is lesser to a more specific cognitive test, such as the Trail Making Tests, which assess cognitive speed and executive functions (285, 286).

Circulating AMH level have been associated to aspects of development in boys. AMH levels are negatively associated with measurements of autism scores in boys (66). AMH also putatively slows the speed of maturation of boys (62), and together may influence neuronal development. This is underscored by animal studies that showed male phenotypic behaviour is associated with the presence of AMH in the prepubescent state (67), which is induced by AMH regulation of specific areas of brain development (287, 288). If AMH does have function in the brain, it might putatively regulate neuronal development during childhood that contributes to the magnitude of cognitive reserve. The cognitive ability of younger humans is predictive of the risk of cognitive decline in older years (289, 290).

Alternatively, AMH may be an indirect marker for cognitive decline. AMH is putatively associated with cardiovascular function (193, 291), and higher levels of circulating AMH are associated with an absence of cardiovascular disease

[Chapter 4A.3.2]. Cardiovascular disease is implicated in the development of cognitive impairment, including vascular dementia and Alzheimer's Disease (292, 293). Circulating AMH level may be a putative biomarker for cognitive impairment associated with vascular pathologies, with this requiring further examination.

AMH may function as a hormone and directly influence brain function. This is similar to the effect of testicular testosterone on cognitive function (294, 295). The ability for AMH to cross the blood brain barrier is unknown, and warrants specific animal studies. AMH itself may act as a paracrine factor, with local production of AMH in the brain being documented (296) and animal studies showing AMH being a neuroprotective factor (127).

7.4.2 Cleavage of AMH as biomarker for weight change and frailty

The extent of proAMH cleavage showed distinct correlation to physical function and weight. The cleavage extent of a hormone may function as a biomarker [Chapter 1.6.1]. Multiple enzymes cleave proAMH in vitro, but there is currently no information about which of the enzyme(s) is physiologically relevant. This limits the ability to draw specific conclusions regrading the mechanism underlying the association between the API and traits.

7.4.3 Testicular hormones exhibit varying association to aspects of ageing

All four testicular hormones decline with ageing, as shown in Chapter 5. However, in this cohort of older men, each testicular hormone exhibit distinct correlation to different traits. Circulating AMH level has statistical significant relationship to cognitive function [Chapter 7.4.1]. Circulating levels of INSL3 is associated to an older's height, which is independent to a person's age. Height typically diminish with ageing as a consequence of reduced vertebral height from osteoporosis (297), and INSL3 is a regulator of bone (11, 219, 298). This provides a direction to investigate for the role of INSL3 in osteoporosis in men, either as a biomarker or a therapeutic target. Circulating level of InhB correlated to the Time-Up-and-Go test, another measure of physical function with relevance to balance, lower limbs muscle strength and coordination. The significance of this correlation is unknown as there are multiple determinants of the TUG test. However when considered in the entirety, the differential profiles of four testicular hormones to different phenotypic traits indicate the need to examine all four testicular hormones in blood, when correlating to biological traits. This pilot study therefore has shown that it is possible to examine all four circulating testicular hormones to a heterogenous group of older men, with the specific interaction between the four of them to a trait requiring further specific studies.

7.4.4 Technical considerations

The use of multiple testings within a domain usually require correction with a statistical technique such as Bonferroni. However, multiple measures within the domains exhibited relationships to each other, thereby significantly reducing the statistical power when applying a correction test such as Bonferroni which applies *a priori* assumption that each variable being tested are independent. For instance, the anthropometric domain includes weight, height and BMI, but BMI being a ratio of weight and height. Partial correlation was therefore applied to correct for known or assumed covariates such as age to circulating hormonal levels and variables within a domain.

This pilot study employed multiple tests for the same domain, using a mixture of continuous testing such as the TUG, and more discrete testing such as the SPPB. Whilst clinical diagnosis require the use of cut-off criterias, greater strength of association may be observed with the use of continuous testing to circulating hormonal values. This pilot study have shown that it is feasible to use both forms of testing to generate further hypotheses for more targeted research.

Depression is associated with cognitive decline, and demonstrated in this pilot study. The results from this Chapter has shown the need to include measurements of depression when correlating hormonal levels to cognitive states due to potential confounding effect of depression.

This study is of cross-sectional nature, which is appropriate as a feasibility study to guide avenues for further research, particularly of a longitudinal design. The associations between circulating testicular hormones to different traits may be acquired with ageing, or reflect baseline level through secular changes.

7.5 Conclusion

This pilot study has shown that it is feasible to correlate circulating AMH levels, and its API, to aspects of age-related function. This generates directions for research to explore for putative functions of AMH, particularly in the area of cognition and physical frailty. The relationship appeared to be distinct to the other testicular hormones. This highlight the need to examine all four testicular hormones when examining for any relationship to traits.

Chapter 8:

Final conclusions

8.1 Introduction

AMH is a testicular hormone in men. Despite its label as a hormone, it is unknown if AMH has any functional significance in the adult male. This thesis began with the premise that circulating AMH level may have functions, and that the functions may not be overt in men. The main objective for this thesis is to advance this argument by setting up the foundations for further research of AMH in men. This thesis has met its objective by documenting basic physiological parameters of circulating AMH. Along the way, the PhD has expanded upon the definition of a hormone, and the concept of testicular endocrine function. The following description is a narrative of this research with its implication to the wider scientific and clinical community.

8.2 Is AMH a hormone?

The understanding of a hormone has expanded vastly in recent years, with the awareness that a hormone may exhibit pleiotropic roles. Insulin-like growth factor is one example, which has dual role as both a hormone (299) and a paracrine factor (300). InhB is well recognised as a testicular hormone, but also a member of the TGFβ superfamily, and subject to its complex regulations and interactions with other signalling molecules [Chapter 1.6.2]. AMH is unlikely to be a classical hormone with overt functions, but it may have cryptic endocrine functions, which are biologically and clinically important. Several lines of evidence advances this argument. AMH is secreted into blood from the testes, and persists into the very old in men. The circulating levels of AMH varies

significantly between men, and exhibit correlation to functional traits in men. These observations are correlative and are insufficient alone to prove function. Nonetheless this thesis provides compelling reason why AMH should be examined in men, as a hormone.

If AMH does have functional significance, it is likely to be a biomarker of the state of the testes. AMH is stable in blood throughout the day, and is independent to the other testicular hormones, including InhB. AMH may putatively reflect the state of spermatogenesis, similar to how circulating AMH is utilised as a biomarker for ovarian reserve in women of reproductive age. Whether total AMH, proAMH or its cleaved form $AMH_{N,C}$ are more accurate reporters of AMH function, or whether they reflect different aspects of AMH function is yet to be determined.

8.3 Establishment of normative range for AMH in men

This thesis has provided the first description of circulating AMH level in older men. The data enables the generation of a normative range for AMH. This can be utilised to identify individuals outside of the normative range for further study into AMH function. All clinical endocrine practice requires the establishment of normal values, and the findings here may be of value for future clinical practice. The use of AMH as a diagnostic marker in the paediatric patient with ambiguous genitalia has been described (301, 302), but this uses a dichotomy of whether AMH is present or absent in blood. The normative values allows a more refined approach for diagnostic purposes. Furthermore, knowledge of normal physiological parameters can guide experimental studies on AMH, which to date has used non-physiological range of AMH dosing, and AMH preparations with undefined proportions of proAMH and $AMH_{N,C}$.

8.4 API is distinct to circulating AMH level and may be a biomarker

The discovery that API is distinct between boys and men, and independent to the circulating level of AMH suggest that API may have significance. This is supported by correlation to functional traits in men. Cleavage activity of a prohormone has been advanced in some studies as putative biomarkers, and this study has certainly advanced that concept. Processing of a hormone following synthesis adds another layer of complexity to the regulation of a hormonal function, and highlights that a simplistic understanding of hormonal function may not be sufficient in today's scientific environment.

8.5 The testes is a complex endocrine organ

The classic understanding of the endocrine testes has primarily been based upon testosterone, including the definition of testicular function. The testes is likely to be a complex organ, with multiple hormones each working independently and in some instances in concert to maintain the male phenotype. Testosterone has a prime role in generating sexual dimorphism, as does AMH, although the other testicular hormones may contribute in subtlier manners. The stability of AMH and INSL3 suggest that they reflect a more basal state of the testes, and less likely to be influenced by sporadic factors. This also challenges the concept that the male phenotype is solely generated by testosterone only. This is particularly pertinent in the understanding of hypogonadism. This thesis has shown that whilst testicular hormones exhibit age-related decline, the phenomenon is occuring independently. Thus there is a spectrum of variability in low levels of testicular hormones, and may reflect different aspects of testicular ageing. Whether one form has more clinical significance than others requires further examination. The thesis therefore sets up the premise that gonadal endocrine function requires the examination of all four testicular hormones, and may in future improve the accuracy of treatment of hypogonadism.

8.6 Future directions

This thesis has generated several lines of enquiries for future research. The determinants of variability in circulating AMH between men is unknown. A long-term longitudinal study of AMH would determine whether levels of AMH established at birth or in childhood is a major determinant to their adult level, or if environmental influences during adulthood plays a bigger role. The association of AMH with functional traits requires more examination in targeted groups with pathologies, such as those with dementia, sarcopenia, or cardiovascular disease. The endogenous half-life of AMH in men is unknown, and its knowledge would guide further studies looking into replacement of AMH in animals and humans to elucidate its function.

8.7 Conclusion

This PhD journey has demonstrated to the author that science is never black and white, but rich in its many hues of discoveries. The exploration of novel research is exciting and equally frustrating, in attempting to change people's perception of a flat earth. The first step of hopefully many more have been cast, to convince the world that men are complex creatures. This thesis adds to the conversation that sometimes, the function of man is influenced by his testes.

Chapter 9:

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