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# The proportion of cleaved anti-Müllerian hormone is higher in serum but not follicular fluid of obese women independently of polycystic ovary syndrome

## BIOGRAPHY

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## KEY MESSAGE

A lower proportion of proAMH is detectable in the serum of obese versus normal weight women independently of polycystic ovary syndrome status. In the follicular fluid, this difference is not found, suggesting that conversion of proAMH into the active isoform occurs in extra-ovarian tissues and is exacerbated in obese individuals.

## ABSTRACT

**Research question:** Does the relative distribution of anti-Müllerian hormone (AMH) isoforms differ between patients depending on their body mass index (BMI) and polycystic ovary syndrome (PCOS) status in serum and follicular fluid?

**Design:** Obese and normal weight patients (PCOS [*n* = 70]; non-PCOS [*n* = 37]) were selected for this case-control study in the serum. Between 2018 and 2019, obese (*n* = 19) and normal weight (*n* = 20) women with or without PCOS who were receiving IVF treatment were included in the follicular fluid study. The bio-banked serums and follicular fluid were tested for total AMH (proAMH and $AMH_{N,C}$ combined) and proAMH using an automatic analyzer. The AMH prohormone index (API = [proAMH]/[total AMH]x 100) was calculated as an inverse marker of conversion of proAMH to $AMH_{N,C}$, with only the latter isoform that could bind to the AMH receptor complex.

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**KEYWORDS**
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**Results:** The API was not significantly different between controls and women with PCOS, whereas obese women had a lower API compared with their normal weight counterparts. Grouping PCOS and controls, a lower API was found in obese versus normal weight women, suggesting a greater conversion of proAMH to $AMH_{N,C}$. The API in the serum was significantly correlated with metabolic parameters. In the follicular fluid, API is not different between obese and normal weight women independently of PCOS and is higher than in the concomitant serum.

**Conclusions:** The proportion of inactive form of AMH in the serum is higher in normal weight versus obese women but not in the follicular fluid, independently of PCOS. The conversion of proAMH into the cleaved isoform is likely to occur in extra-ovarian tissues and to exacerbate in obese individuals.

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex reproductive endocrine disorder, affecting up to 20% of women of reproductive age and representing the primary cause of anovulatory infertility worldwide (*Teede et al., 2018*). In about 50% of cases, PCOS associates with metabolic disturbances, such as obesity, type 2 diabetes mellitus, cardiovascular disease and other comorbidities (*Dumesic et al., 2015*; *Escobar-Morreale, 2018*). Moreover, obesity itself increases the symptoms of PCOS, such as ovulation disorders or hyperandrogenism.

A frequent hallmark found in women with PCOS, is an elevation of anti-Müllerian hormone (AMH) serum levels, which have been reported to be two to threefold higher than in women with healthy ovaries (*Cook et al., 2002*; *Pigny et al., 2003*). The severity of the PCOS phenotype correlates with AMH production, which is higher in patients with anovulatory than in ovulatory PCOS (*Pellatt et al., 2007*; *Catteau–Jonard et al., 2012*; *Abbara et al., 2019*). Clinically observed correlations between enhanced basal levels of AMH and PCOS have led to a potential physiological role for AMH in 'follicular arrest' in women with PCOS (*Dewailly et al., 2014*). In this pathology, enhanced numbers of primary and small secondary follicles secrete abnormally high levels of AMH, which associates with excess androgen production or secretion, and leading to arrested follicular maturation (*Dewailly et al., 2014*).

Anti-Müllerian hormone is a glycoprotein synthesized as a homo-dimeric prohormone of 140 kDa (composed of two monomers of 70 kDa), which undergoes a proteolytic cleavage at monobasic sites to become biologically active (*Pepinsky et al., 1988*; *di Clemente et al., 2010*). The cleavage of AMH gives rise to a 110 kDa N-terminal homodimer formed by two 57 kDa subunits ($AMH_N$) and a 25 kDa active C-homodimer composed of two identical 12.5 kDa ($AMH_C$) subunits. These two homodimers remain associated in a non-covalent complex ($AMH_{N,C}$) (*Nachtigal and Ingraham, 1996*; *Pepinsky et al., 1988*). Although having no intrinsic activity, $AMH_N$ has the role of amplifying the biological activity of the $AMH_C$ (*Wilson et al., 1993*). $AMH_{N,C}$ and its pro-hormone (proAMH) are found in blood but there is little evidence for the presence of free $AMH_C$ (*Pankhurst and McLennan, 2013*). This suggests that circulating cleaved $AMH_{N,C}$ levels represent the receptor-competent isoform available for a hormonal function in a variety of tissues and organs expressing the exclusive binding-receptor AMHR2 (*Barbotin et al., 2019*).

Several studies point to AMH as a useful marker for the diagnosis of PCOS; however, challenges to the use of AMH to diagnose PCOS have been fully reviewed in *Teede et al. (2018)*. *Abbara et al. (2019)* recently suggested that AMH has potential as a biomarker for diagnosing PCOS, outperforming antral follicle counts, and greater elevations in AMH were associated with a more certain diagnosis of PCOS. Because of the variability in levels using first-generation and less reliable assays, and the lack of an international standard, AMH has not yet been incorporated into the diagnostic criteria for PCOS (*Teede et al., 2018*).

In recent years, novel approaches and analytical assays have been used (*Pankhurst et al., 2017*; *Wissing et al., 2019*), which aim to determine whether the ratios of proAMH or $AMH_{N,C}$ in serum are altered in women with PCOS relative to women without PCOS, and to understand if different AMH-related isoforms can be used to predict PCOS. The results of those studies, however, are conflicting, either reporting differences in the mean proAMH–$AMH_{N,C}$ ratio in the PCOS group compared with the unaffected control group (*Pankhurst et al., 2017*) or reporting no differences in the relative distribution of the AMH isoforms between those groups (*Wissing et al., 2019*). We hypothesized that part of the discrepancies could be due to the heterogeneity of the disorder, and that stratifying women with and without PCOS based on their metabolic phenotype (body mass index [BMI]) could better help to pin-point eventual differences in AMH cleavage. These differences in AMH cleavage could lead to relative higher levels of active AMH in the serum, which could contribute to the existence of different PCOS phenotypes.

The aims of this study were, therefore, to determine if the relative distribution of AMH isoforms differ from one patient to another depending on their BMI and PCOS status, and to investigate whether differences in the proportions of AMH isoforms are caused by increased proAMH cleavage occurring in the follicular fluid or in the serum.

## MATERIALS AND METHODS

### Population for early follicular phase serum study

Patients referred to our department at an academic hospital for hyperandrogenemia, oligo-anovulation, infertility, or all three, were identified from a database that included clinical, hormonal and ultrasound features, which were consecutively recorded between January 2014 and December 2018. A total of 70 women with PCOS (42 obese; BMI ≥30 kg/$m^2$ and 28 normal weight; BMI ≤25kg/$m^2$) and 37 controls (16 obese and 21 normal weight) were selected retrospectively, as described below. Patients were included in the PCOS group according to a modified Rotterdam classification (*Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004*), i.e. presence of at least two out of three of the following items: hyperandrogenism (clinical or biological), as previously described (*Peigné et al., 2018*); oligo-anovulation, i.e. oligomenorrhoea or amenorrhoea; presence of polycystic

ovarian morphology at ultrasound, with an ovarian area 5.5 $cm^2$ or greater, a follicle number per ovary 19 or more, or both, according to our inhouse thresholds, as previously reported (*Dewailly et al., 2011*).

Controls were referred to our department for infertility caused by tubal, sperm abnormality, or both. None of these women had any component of the Rotterdam classification: they had regular cycles, no hyperandrogenism and normal ovaries at ultrasound. All medications known to affect metabolism, e.g. metformin, or ovarian function, e.g. oral contraceptive pill, had to be stopped at least 3 months before blood tests and ultrasound.

### Investigations

During the medical examination, age, height, weight and waist circumferences were registered. Blood sampling was carried out in the early follicular phase, i.e. between cycle days 2 and 5; either spontaneous or induced by dydrogesterone). Immunoassay was used to measure FSH, oestradiol, LH, total testosterone, fasting serum insulin levels using an automatic analyzer (Architect) (Abbott Laboratories, Abbott Park, IL, USA). Sex hormone binding globulin (SHBG) levels were measured by immunoassay using the Immulite analyzer (Siemens, Munich, Germany). Androstenedione levels were determined by liquid chromatography–mass spectrometry. Low limit of quantitation and intra- and inter-assay variation are presented in Supplementary Table 1. Biochemical tests (glucose, lipid levels) were carried out in the central biochemistry department of the hospital using routine assays on Roche analyzers.

### Population for follicular fluid and concomitant serum study

Between October 2018 and January 2019, women undergoing IVF ovarian stimulation were included prospectively either in the obese (PCOS or control) or the normal weight group (PCOS or control). Selection criteria were identical to those described earlier. Follicular fluid collected after oocyte retrieval (as described below), and serum, retrieved at the last hormonal check-up (2–3 days before oocyte retrieval and frozen at –20° after hormonal analysis), were used for AMH measurements.

### Follicular fluid samples

All follicles wider than 12 mm were retrieved during the oocyte retrieval procedure. After oocyte isolation for IVF procedure, follicular fluid was collected, centrifuged at 1680 g for 5 min to remove cells and frozen at –80° until analysis.

### Anti-Müllerian hormone measurements

Routinely, fully automated Access DxI sandwich chemoluminescent immunoassays (Beckman Coulter, Brea, CA, USA) were used to measure serum AMH levels. The standard used in the Access DxI assay is the human recombinant 140 kDa AMH. Its limit of detection is around 0.47 pmol/l (Pigny, unpublished) and its measurement range is 0.47–150 pmol/l (*van Helden and Weiskirchen, 2015*) (Supplementary Table 1). The Access DxI assay used the same antibodies pair as the AMH Gen II ELISA: a capture antibody binding C-terminal part of AMH and a detection antibody initially thought to be directed against the C-terminal of AMH but which also required the N-terminal part of AMH for correct detection (*Kevenaar et al., 2006*; *Pankhurst et al., 2014*). On the basis of these features, it can be assumed that the Access DxI assay recognizes in the biological samples both the pro-AMH (uncleaved) and the $AMH_{N,C}$ (receptor competent-active). To fix this point, four serums were spiked with 10–25 ng/ml of recombinant $AMH_C$ (25 kDa, R&D Systems, MIS 1737). Results showed the absence of cross-reactivity of $AMH_C$ in this assay (Supplementary Table 2). It was, therefore, concluded that the only molecular AMH forms detected in the serum by DxI Automatic analyzer were proAMH + $AMH_{N,C,}$ which were defined as total AMH (Supplementary Figure 1A).

### Specific measurement of anti-Müllerian hormone molecular forms in serum and follicular fluid

A specific assay for proAMH measurement was built for Beckman Coulter DxI automatic analyzer based on a previous protocol designed with the AMH Gen II ELISA (*Pankhurst and McLennan, 2016*) and used in the leftover serum or follicular fluid of the patients. This experimental protocol included a deoxycholate (DOC) treatment step of the serum, which leads to the dissociation of the non-covalent link between N-terminal and C-terminal parts of $AMH_{N,C,}$ which prevents binding of the detection antibody. The ProAMH is unaffected by the DOC treatment as the molecule is uncleaved (Supplementary Figure 1B).

With the Gen II ELISA, the DOC treatment step was carried out between the capture and detection steps, which is feasible in a manual assay but not possible with an automatic assay. Therefore, the samples were pre-treated before assaying AMH with the analyzer. On the basis of the study by *Pankhurst and McLennan (2016)*, independent experiments were conducted to set up DOC concentration, incubation time and temperature. The final protocol is described below.

Two 150-µl aliquots were made from each sample (bio-banked serum or follicular fluid, stored at –80°). Phosphate buffered saline (PBS) (0.02 M, pH = 6.8, 37°C) was added in one aliquot (control) whereas sodium deoxycholate in PBS at 37°C (final concentration 0.23%, i.e. 2333 µg/ml, corresponding to nearly 6000 times more than the endogenous serum DOC concentration, was added in the second aliquot). Aliquots were incubated at 37°C for 15 min before being spiked again with DOC or PBS solution and further incubated for 15 min at 37°C. Final volumes of both aliquots were 300 µl, which allow a direct measurement with the Beckman Coulter Access DxI automatic analyzer. Control aliquot with PBS allows the measurement of total AMH in the sample, whereas aliquot with DOC treatment allows the measurement of proAMH only (Supplementary Figure 1).

The AMH prohormone index (API) (*Pankhurst and McLennan, 2016*)) was calculated as a surrogate marker of conversion of proAMH to $AMH_{N,C}$; API = [proAMH]/[total AMH] x 100.

### Validation of the specific proAMH assay

As previously described (*Pankhurst and McLennan, 2013*), an aliquot of recombinant human AMH was treated with furin to convert proAMH to $AMH_{N,C.}$ By western blotting, it was observed that the untreated preparation (recombinant human AMH) mainly contains proAMH (around 77% after densitometric analysis), whereas the furin-treated preparation mainly contains $AMH_{N,C}$ (around 60%) (Supplementary Figure 2A and 2B). A total of 20-µg of proteins were loaded in each well composed of 4–12% Tris-glycine bisacrylamide gels at 120 volts for 15 min and then 150 volts for 75 min using the Xcell Surelock Mini-Cell system (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to a 0.45-µm nitrocellulose membrane (Whatman PLC,

Maidstone, UK) at 30 volts for 1 h 30 min on ice. Membranes were blocked in TBS-T (tri-buffered saline, 0.05% Tween 20) + 5% milk for 1 h at room temperature, followed by incubation with 0.1 ng/ml primary antibody (AF2748) (R&D Systems, Minnneapolis, MN, USA) in TBS-T plus 5% bovine serum albumin overnight at 4°C. The next day, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (IRDye680 Donkey anti-goat immunoglobulin G antibody) (LI-COR Biosciences, Lincoln, NE, USA) diluted at 1:10000 in TBS-T plus 5% milk for 1 h at room temperature.

Finally, the membranes were incubated in SuperSignal West Pico PLUS (ThermoFisher, Waltham, MA USA) and revelation was carried out in a dark room using X-ray films. Fiji image analysis software was used for quantification

Both preparations were measured after dilution using our specific assay for proAMH. As expected, addition of DOC induced a strong decrease of the AMH concentration in the furin-treated preparation. This demonstrated that DOC treatment reduces the assayed AMH value in a manner that is consistent with dissociation of the $AMH_{N,C}$ complex (Supplementary Figure 2C). The assayed AMH values after DOC pre-treatment were not modified by the lipid profile of the dilution medium (serum of menopausal women with or without dyslipidaemia, or in the standard of the Access DxI assay, which does not contain AMH or lipids) (Supplementary Figure 2D).

No difference in total AMH levels measured with the DxI Analyzer was observed between proAMH (either standard of the Access DxI assay or recombinant human AMH provided by *Pankhurst and McLennan (2016)*) treated by DOC versus PBS buffer (Supplementary Table 3), thus confirming that proAMH is unaffected by the DOC treatment.

### Statistical analysis

SPSS software 22.0 (SPSS Inc.) (Chicago, IL, USA) was used for all statistical analyses. Results were expressed as medians, with fifth to 95th or 10th to 90th percentiles for continuous variables and by the frequencies and percentages for categorical variables. Comparisons between two independent groups were conducted using the Mann–Whitney U test and between dependent groups (for paired comparison between follicular fluid and serum results) using the Wilcoxon signed rank test. Comparisons between four groups were conducted using the analysis of variance test with post-hoc pairwise comparisons between obese and normal weight controls, and obese and normal weight PCOS after applying Bonferroni correction. The correlations between the various parameters were expressed as Spearman's correlation coefficient (r). $P < 0.05$ was considered to be significant and a r absolute value ≥0.4 was considered to be strong.

### Ethics

For the first part of the study, all patients had previously provided informed consent for the use of their clinical, hormonal, ultrasound records and leftover serum. For the second part of the study, all patients provided informed consent for the use of their follicular

**TABLE 1 POPULATION CHARACTERISTICS IN THE EARLY FOLLICULAR PHASE SERUM STUDY**

| | Control (*n* = 37) | | PCOS (*n* = 70) | | *P*-value |
|---|---|---|---|---|---|
| | Normal weight (*n* = 21); median (5–95) | Obese (*n* = 16) ; median (5–90) | Normal weight (*n* = 28); median (5–95) | Obese (*n* = 42) ; median (5–95) | |
| Age, years | 32.0 (22.4–38)[c,d] | 31.5 (25–35.3)[c] | 23 (16.3–33)[a,b] | 27 (19.1–38.0)[a] | <0.001 |
| Weight, kg | 59.0 (47.1–65.4)[b,d] | 94.2 (81.0–129.1)[a,c] | 55.8 (44.4–72.7)[b,d] | 91.0 (74.0–123.5)[a,c] | <0.001 |
| BMI, kg/m$^2$ | 20.7 (16.4–24.9)[b,d] | 33.7 (29.0–43.85)[a,c] | 20.5 (17.1–25.9)[b,d] | 34.0 (30.0–42.95)[a,c] | <0.001 |
| Waist circumference, cm | 72.0 (58.5–78.9)[b,d] | 103.5 (79.5–133.2)[a,c] | 77 (62.8–100)[b,d] | 107.0 (95.1–129.9)[a,c] | <0.001 |
| Testosterone, ng/ml | 0.22 (0.13–0.41)[c,d] | 0.31 (0.16–0.41) d | 0.38 (0.17–0.64)[a] | 0.45 (0.21–0.88)[a,b] | <0.001 |
| Androstendione, ng/ml | 0.94 (0.5–1.58)[c,d] | 0.77 (0.42–1.72)[c,d] | 1.29 (0.74–2.87)[a,b] | 1.43 (0.64–3.04)[a,b] | <0.001 |
| Oestradiol, pg/ml | 36.0 (18.8–67.6) | 34.0 (17.0–60.5) | 34.0 (16.2–94.4) | 42 (28.1–64.8) | 0.565 |
| LH, IU/l | 4.1 (1.53–12.7) | 3.05 (1.5–5.5)[c,d] | 5.4 (2.4–17.2)[b] | 5.2 (2.2–11.7)[b] | 0.005 |
| FSH, IU/l | 5.5 (3.5–10.2) | 5.35 (4–7.67) | 5.1 (2.9–6.9) | 5.0 (3.2–8.2) | 0.135 |
| SHBG, pmol/l | 70.4 (40.7–103.8)[b,c,d] | 36.3 (17.2–65.75)[a] | 47.5 (24.9–96.8)[a,d] | 28.8 (10.1–64.2)[a,c] | <0.001 |
| Fasting glycaemia, g/l | 0.77 (0.71–0.9)[d] | 0.86 (0.7–0.99) | 0.81 (0.69–0.94) | 0.85 (0.72–1.13)[a] | 0.013 |
| Fasting insulinaemia, mIU/l | 3.4 (0.9–13.0)[d] | 5.6 (2.5–12.22)[d] | 4.6 (2.0–12.7)[d] | 11.6 (3.1–24.5)[a,b,c] | <0.001 |
| Total cholesterol, g/l | 1.8 (1.1–2.4) | 1.66 (1.29–2.36) | 1.74 (1.19–2.6) | 1.89 (1.46–2.65) | 0.182 |
| HDL cholesterol, g/l | 0.58 (0.38–0.77)[b,d] | 0.48 (0.32–0.61)[a] | 0.56 (0.376–0.78)[d] | 0.45 (0.29–0.58)[a,c] | <0.001 |
| LDL cholesterol, g/l | 1.03 (0.57–1.58) | 1.06 (0.66–1.59) | 0.98 (0.58–1.74) | 1.26 (0.78–1.81) | 0.183 |
| Triglycerides, g/l | 0.59 (0.36 -1.42)[d] | 0.86 (0.44–1.65) | 0.65 (0.33–1.65)[d] | 1.1 (0.58–2.98)[a,c] | <0.001 |
| Total AFC both ovaries | 22 (8.5–35)[c,d] | 21 (6–30.1)[c,d] | 52 (14.2–123)[a,b] | 55 (24.3–99.0)[a,b] | <0.001 |

[a] Statistically different from normal weight controls.
[b] Statistically different from obese controls.
[c] Statistically different from normal weight patients with PCOS.
[d] Statistically different from obese patients with PCOS.
AFC, antral follicle count; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCOS, polycystic ovary syndrome; SHBG, sex hormone binding globulin.

fluid and leftover serum. The Institutional Review Board of the University Hospital granted unrestricted approval to the anonymous use of clinical, hormonal, ultrasound records and biologic collection from all patients (reference DC-2008-642, approved July 2009).

## RESULTS

### Molecular forms of anti-Müllerian hormone in early follicular phase serum

One hundred and seven women were selected for this first part of the study: 70 women with PCOS (42 obese and 28 normal weight) and 37 controls (16 obese and 21 normal weight). The main clinical, hormonal, metabolic and ultrasound characteristics of the study population are presented in TABLE 1 and Supplementary Table 4.

Total AMH and proAMH levels were higher in women with PCOS, independently of their metabolic features (BMI and insulin resistance, indirectly evaluated by waist circumference, SHBG levels and fasting insulinaemia), compared with controls: respectively for total AMH (PCOS: 47.4 pmol/l, 95% CI 26.5 to 133.5 versus controls: 17.2 pmol/l, 95% CI 7.8 to 35.7; $P < 0.001$) and proAMH (PCOS: 19.6 pmol/l, 95% CI 7.5 to 55.3 versus controls: 6.6 pmol/l, 95% CI 2.6 to 15.9; $P < 0.001$).

The AMH prohormone index (API = [proAMH]/[total AMH] x 100) was then used as a surrogate marker of conversion of proAMH to $AMH_{N,C}$, i.e. low index indicating increased proAMH conversion into receptor-competent isoform. No difference in the API was found between patients with and without PCOS when we stratified the population study group based only on PCOS diagnosis (PCOS: 34.6%, 95% CI 28.6 to 48.3 versus controls 38.2%, 95% CI 28.7 to 51.0; $P = 0.8$).

When patients were grouped by BMI, obese women compared with their normal weight counterparts in both control and PCOS groups had an API significantly lower (respectively for controls with normal weight (39.02%, 95% CI 32.2 to 54.5) versus obese (34.6%, 95% CI 28.12 to 42.48; $P = 0.015$) and for women with PCOS with normal weight (42.4%, 95% CI 31.5 to 48.6) versus obese (35.22%, 95% CI 27.63 to 49.42; $P < 0.001$), suggesting a greater conversion of proAMH to $AMH_{N,C}$ (FIGURE 1 and Supplementary Table 5). Serum total AMH and proAMH levels were not significantly different

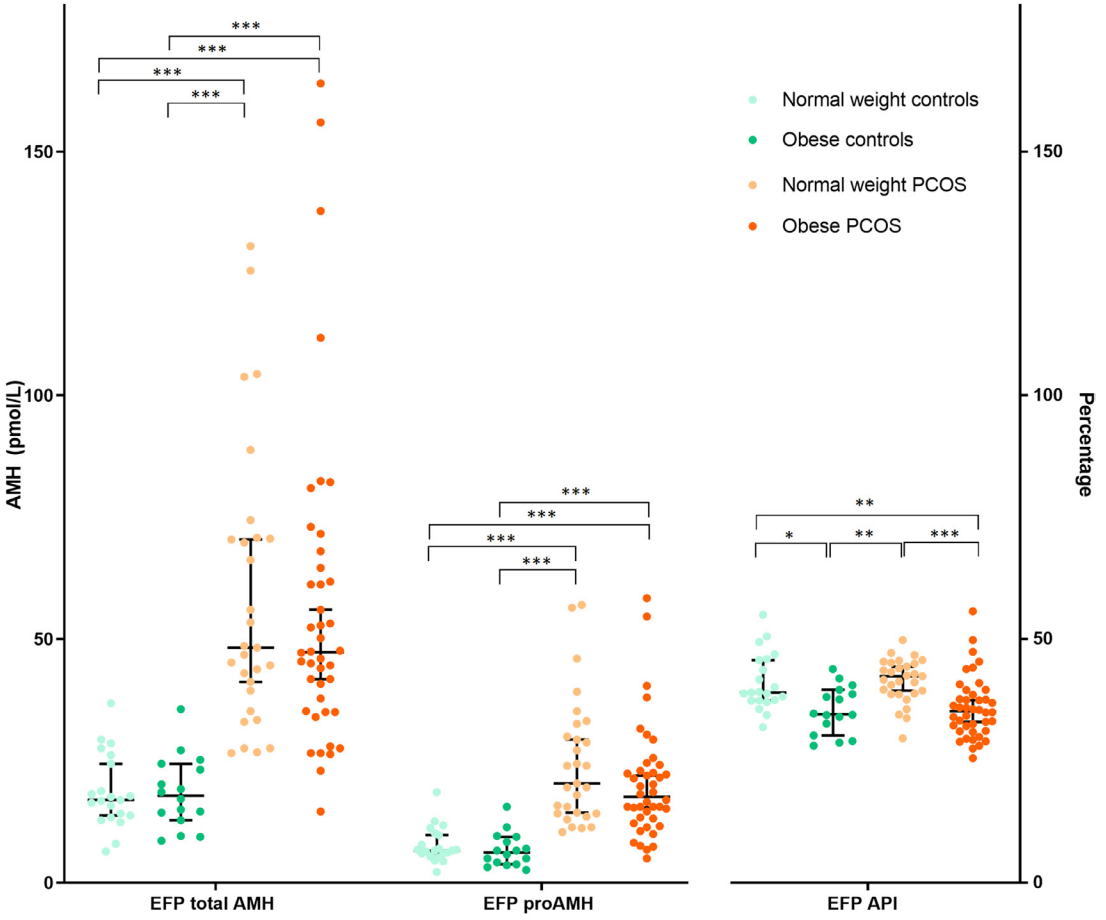


FIGURE 1 Anti-Müllerian hormone (AMH) molecular forms in the early follicular phase (EFP) serum: women with polycystic ovary syndrome (PCOS) versus controls grouped by body mass index (BMI). Total AMH (pmol/l), AMH proprotein (proAMH) (pmol/l) and API = (proAMH/totalAMH) x100 in women with PCOS and controls, according to their BMI (obese = BMI ≥30.0 kg/m$^2$) and normal weight (BMI ≤25.0 kg/m$^2$). Green dots: control women ($n = 37$) with lighter green dots, normal weight control women ($n = 21$) and darker green dots, obese control women ($n = 16$); orange dots: women with PCOS ($n = 70$) with lighter orange dots, normal weight women with PCOS ($n = 28$) and darker orange dots, obese women with PCOS ($n = 42$). Data are presented as medians with interquartile ranges.
*$P = 0.015$, ** $P = 0.004$, *** $P < 0.001$. Groups that are not linked by a line did not exhibit statistically significant differences.

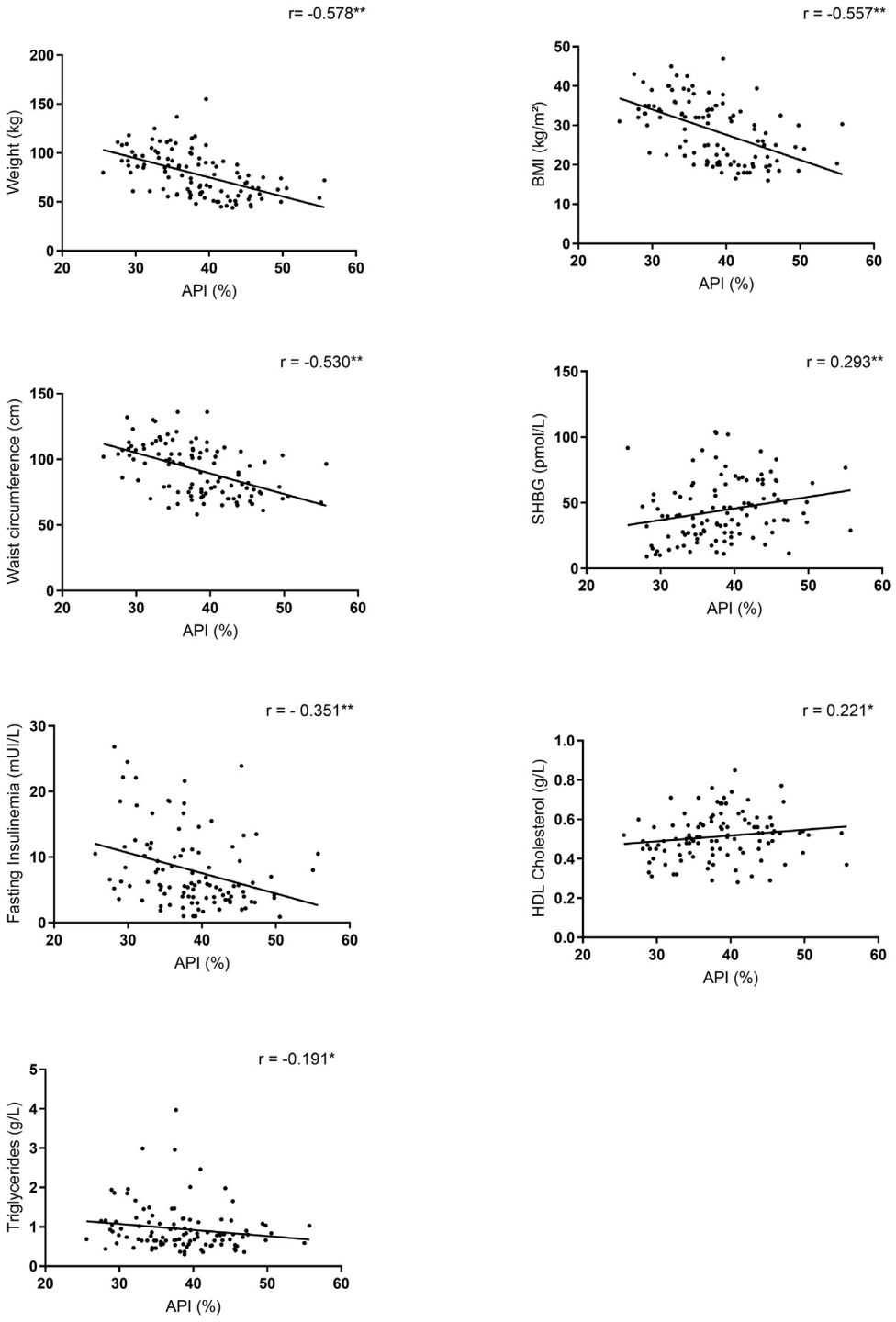


**FIGURE 2** Correlation of anti-Müllerian hormone (AMH) prohormone index (API) in the early follicular phase serum with metabolic parameters. Spearman correlation; API, (proAMH/totalAMH) x 100l; EFP, early follicular phase; SHBG, sex hormone binding globulin. *$P < 0.05$, **$P < 0.01$, r = correlation coefficient.

between obese and normal weight women in each group (FIGURE 1 and Supplementary Table 5), respectively, in control group for serum total AMH levels ($P = 1.0$) and for serum proAMH level ($P = 1.0$) and in the women with PCOS group for serum total AMH ($P = 1.0$) and for serum proAMH level ($P = 0.468$).

Possible interactions between the API and pathophysiological features of PCOS were then investigated. When patients with PCOS were grouped, a significantly lower API was found in obese compared with normal weight women (34.9%, 95% CI 28.1 to 47.5 versus 41.2%, 95% CI 32.8 to 50.2, respectively; $P < 0.001$), whereas total AMH and proAMH levels were not significantly different ($P = 0.393$ and $P = 0.945$, respectively).

In the whole population ($n = 107$), the API negatively correlated to some metabolic parameters: weight ($r = -0.578$; $P < 0.01$), BMI ($r = -0.557$; $P < 0.01$), waist

**TABLE 2 CORRELATION OF PROAMH RATIO IN THE SERUM AND FOLLICULAR FLUID WITH CLINICAL, HORMONAL, ULTRASONOGRAPHIC AND METABOLIC PARAMETERS**

| | Serum early follicular phase API rho Spearman | Follicular fluid API rho Spearman |
|---|---|---|
| Age | –0.16 | 0.088 |
| Weight | –0.578[a] | –0.192 |
| BMI | –0.557[a] | –0.094 |
| Waist circumference | –0.530[a] | –0.262 |
| Testosterone | –0.168 | –0.052 |
| Androstendione | –0.073 | –0.080 |
| Oestradiol | –0.025 | 0.092 |
| LH | 0.042 | –0.128 |
| FSH | 0.134 | 0.278 |
| SHBG | 0.293[a] | 0.650 |
| Fasting glycaemia | –0.121 | –0.285 |
| Fasting insulinemia | –0.351[a] | –0.550 |
| Total cholesterol | –0.056 | |
| HDL cholesterol | 0.221[b] | |
| LDL cholesterol | –0.092 | |
| Triglycerides | –0.191[b] | |
| Total AFC both ovaries | 0.003 | –0.062 |
| Serum total AMH at the end of stimulation | | –0.197 |
| Serum proAMH at the end of stimulation | | –0.136 |
| Serum API at the end of stimulation | | –0.283 |

[a] $P < 0.01$ (bilateral).
[b] $P < 0.05$ (bilateral).
AFC, antral follicle count; AMH, anti-Müllerian hormone; API, AMH prohormone index; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SHBG, sex hormone binding globulin.

circumference (r = –0.530; $P < 0.01$), triglycerides level (r = –0.191; $P < 0.05$) and fasting insulinemia (r = –0.351; $P < 0.01$) (FIGURE 2 and TABLE 2). Moreover, the API was found to be positively correlated with SHBG (r = 0.293; $P < 0.01$), and HDL cholesterol level (r = 0.221; $P < 0.05$) (FIGURE 2 and TABLE 2) No significant correlation was found with serum androgens, LH, LDL cholesterol or total cholesterol levels (TABLE 2).

### Molecular forms of anti-Müllerian hormone in follicular fluid and serum of patients at the end of ovarian stimulation

ProAMH is known to be secreted by granulosa cells and thus released into the follicular fluid before reaching the serum (*Campbell et al., 2012*). An attempt was made to determine whether the cleavage of this protein occurs within the ovaries or in the serum by analysing the API in those samples.

Because the API in early follicular phase serum was only different between obese and normal weight women, this second part of the study focused on 39 patients: 20 normal weight women (including 10 with PCOS) and 19 obese women (including nine with PCOS). The proportion of patients with PCOS in both groups (obese and normal weight) was not different ($P = 1.0$). The main clinical, hormonal, metabolic, ultrasound and ovarian stimulation features of this second study population are shown in Supplementary Table 6.

Total AMH and proAMH were measured in the follicular fluid retrieved after oocyte retrieval as well as in the concomitant sampled serum of each woman. The total AMH and the proAMH were comparable in normal weight versus obese women when comparing the protein amount either in the follicular fluid or in the serum (Supplementary Table 7); respectively in the follicular fluid for total AMH (18.9 pmol/l, 95% CI 7.2 to 56.4 versus 21.4 pmol/l, 95% CI 6.6 to 86; $P = 0.627$) and for proAMH (13.1 pmol/l, 95% CI 4.9 to 43.4 versus 15.4 pmol/l, 95% CI 3.6 to 76; $P = 0.879$. When the API was analysed, no significant differences were found in the follicular fluid between the two study groups ($P = 0.478$) (FIGURE 3A and Supplementary Table 7), whereas the API was significantly reduced in the concomitant serum of obese women compared with normal weight women ($P = 0.001$) (FIGURE 3B and Supplementary Table 7). Interestingly, in both normal weight and obese women, the API was significantly higher ($P < 0.001$) in the follicular fluid than in matched serum samples (FIGURE 3A and FIGURE 3B) (Supplementary Table 7), thus suggesting that most of the cleavage of proAMH occurs in the general circulation rather than in the ovaries.

Lastly, no correlation was found between the API in follicular fluid and any clinical, hormonal, metabolic, ultrasonographic or ovarian stimulation parameters studied (TABLE 2) (univariate analysis in the whole population [$n = 39$]).

## DISCUSSION

In this study, we found that the proportion of the inactive form of AMH in the serum is higher in normal weight compared with obese women (greater API value), independently of PCOS. Consistent with this, obese women have a lower API than women with normal weight, pointing to a more pronounced AMH cleavage in obesity. In contrast to our findings, *Pankhurst et al. (2017)* described a minor reduction in the ratio between proAMH (inactive) over total AMH in PCOS women. This study involved a heterogeneous study group in which all patients were overweight but the PCOS group had a higher incidence of obesity. Our data are in keeping with the view that women with PCOS have a heterogenous spectrum of phenotypic presentation highly susceptible to several variables, such as age, BMI, diet composition, to name a few, and that future areas of research should take the different clinical features of PCOS into account.

Our data confirm and expand upon a recent study showing that the ratio of AMH isoforms do not differ between patients with PCOS and controls (*Wissing et al., 2019*), thus implying a similar distribution of uncleaved (inactive) and cleaved AMH in the two study groups. *Wissing et al. (2019)* analysed a study group composed of women who were either normal weight, slightly overweight,

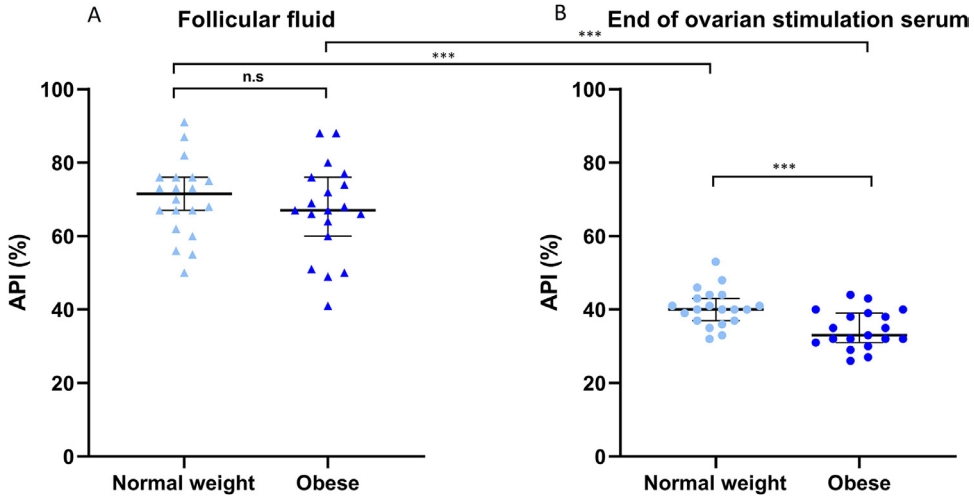


**FIGURE 3** Proportion of anti-Müllerian hormone (AMH) molecular forms in the follicular fluid and in the end of ovarian stimulation serum (Serum): normal weight versus obese women. The AMH prohormone index (API) (API [proAMH/totalAMH] x 100) values in follicular fluid (A) and serum at the end of ovarian stimulation for IVF (B) of the same obese or normal weight women. Light blue, normal weight women ($n$ = 20); dark blue, obese women ($n$ = 19); triangles, follicular fluid (A); circles, end of ovarian stimulation serum (B). Data are presented as medians with interquartile ranges. ***$P \leq 0.001$. For follicular fluid (normal weight) versus follicular fluid (obese), $P = 0.478$.

had PCOS and were taking metformin, or all three. They reported a negative correlation between metabolic parameters and the proportion of inactive AMH form in the serum. Here, we confirmed the correlation results found in *Wissing et al. (2019)*, with an even greater coefficient of correlation between the API and BMI (r = –0.557) and waist circumference (r = –0.530) and demonstrated that a greater processing of AMH in the circulation does not associate with PCOS but rather with a severe metabolic disturbance, such as obesity.

In this study, we found significantly less conversion of proAMH into the cleaved isoform ($AMH_{N,C}$) in the follicular fluid compared with the concomitant serum of the study participants, which was independent of their weight and disease state (PCOS or not). In contrast to a previous study (*Pierre et al., 2016*), which described a higher cleavage of AMH in the follicular fluid in women with PCOS compared with controls, we did not find a difference of cleavage of AMH at the follicular fluid level between women with PCOS and controls. Part of this discrepancy could be explained by the smaller number of patients recruited in the study by *Pierre et al. (2016)* compared with the present work and by the different analytical methods used to measure AMH isoforms in the two studies.

Our data suggest that the cleavage of proAMH only partly occurs at the follicular level and that most of the cleavage of proAMH mainly occurs in extra-ovarian tissues. Subtilisin/kexin-like proprotein convertases such as furin (PCSK3) or PCSK5 are thought to be responsible for AMH cleavage (*Nachtigal and Ingraham, 1996*). Plasmin is also a candidate for proAMH cleavage because, *in vitro,* recombinant proAMH can be cleaved by plasmin (*Pepinsky et al., 1988*). Some investigators have described that proAMH cleaving proteases are found in vascular tissues (*McLennan and Pankhurst, 2015*). The in-vivo cleavage of proAMH remains largely unknown and it likely occurs at several levels and tissues. Interestingly, serum furin levels are known to be modulated by inflammatory conditions, such as those found in metabolic or cardiovascular diseases (*Fathy et al., 2015*; *Ren et al., 2017*). Further studies are required to assess the in-vivo molecular nature of the AMH cleavage, the exact anatomical sites of AMH conversion and whether this could be influenced by the metabolic status of the individual.

An emerging hypothesis proposes that AMH may play several key extra-gonadal roles, acting across multiple sites along the hypothalamic–pituitary–gonadal axis (*Barbotin et al., 2019*). This raises the hypothesis that AMH could act as an endocrine signal, virtually operating on any cell type expressing its exclusive binding receptor AMHR2 throughout the body. Indeed, AMH and AMHR2 expression have been reported in prostate, lungs (*Gustafson et al., 1993*) and several other organs, including the brain (*Wang et al., 2005*; *2009*; *Lebeurrier et al., 2008*; *Cimino et al., 2016*; *Malone et al., 2019*;) and the pituitary (*Garrel et al., 2016*). Moreover, metabolic organs, such as liver and pancreas as well as the hypothalamic arcuate nucleus, also express AMHR2 (*Cimino et al., 2016*; *Segerstolpe et al., 2016*), suggesting that potentially AMH could act on those tissues.

The present finding should be interpreted with caution regarding the study sample size as no formal size calculation was conducted to determine the appropriate number of patients and control. In a posterior power calculation, we calculated that, with our study sample size (37 controls and 70 women with PCOS), we could detect with a 80% power (two-tailed test at 5% significance level), an effect size of 0.57 (expressed as standardized mean difference and interpreted as medium effect size *[Cohen, 1992]*).

In conclusion, this study shows that circulating AMH isoform concentrations cannot be used to improve the precision of PCOS phenotype diagnosis. The novelty of this study, however, relies on the findings that a higher proportion of cleaved, most likely bioactive, AMH ($AMH_{N,C}$) is detectable in the serum of obese women compared with women of normal weight; and a greater cleavage of proAMH to $AMH_{N,C}$ is likely occurring in extra-ovarian tissues and exacerbated

in obese women. This study opens new avenues of investigation aimed at addressing novel roles of AMH in the context of metabolic disturbances associated with reproductive alterations.

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## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.07.020.

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