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Maëliiss Peigné, Pascal Pigny, Michaël W. Pankhurst, Elodie Drumez, Anne Loyens, et al.. The proportion of cleaved anti-Müllerian hormone is higher in serum but not follicular fluid of obese women independently of polycystic ovary syndrome. *Reproductive BioMedicine Online*, 2020, 41, pp.1112 - 1121. 10.1016/j.rbmo.2020.07.020 . hal-03493031

HAL Id: hal-03493031

<https://hal.science/hal-03493031>

Submitted on 15 Dec 2022

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1 **The proportion of cleaved Anti-Müllerian Hormone (AMH) is higher in the serum but**
2 **not in the follicular fluid of obese women independently of Polycystic Ovary Syndrome**
3 **(PCOS)**

4 Maëliiss Peigné^{1,2,3§}, Pascal Pigny^{1,4}, Michaël W. Pankhurst⁵, Elodie Drumez^{6,7}, Anne
5 Loyens¹, Didier Dewailly^{1,3}, Sophie Catteau-Jonard^{1,3*}, Paolo Giacobini^{1*§}

6

7 ¹ Univ. Lille, Inserm, CHU Lille, U1172 - LilNCog - Lille Neuroscience & Cognition, F-59000 Lille,
8 France.

9 ² AP-HP- Université Sorbonne Paris-Nord, Service de Médecine de la Reproduction et Préservation de
10 la Fertilité, Hôpital Jean Verdier, F-93143 Bondy, France.

11 ³ CHU Lille, Service de Gynécologie Médicale, Hôpital Jeanne de Flandre, F-59000 Lille, France

12 ⁴ CHU Lille, Service de Biochimie et Hormonologie, Centre de Biologie Pathologie, F-59000 Lille,
13 France

14 ⁵ Department of Anatomy, School of Biomedical Sciences, University of Otago, Dunedin, New
15 Zealand

16 ⁶ Univ. Lille, CHU Lille, ULR 2694 - METRICS : Évaluation des technologies de santé et des
17 pratiques médicales, F-59000 Lille, France

18 ⁷ CHU Lille, Department of Biostatistics, F-59000 Lille, France HU Lille, Unité de Méthodologie -
19 Biostatistique et Data Management, F-59000 Lille, France

20

21 *These authors contributed equally to this work

22

- 23 § Correspondence should be addressed to: Paolo Giacobini (paolo.giacobini@inserm.fr) or Maëli
- 24 Peigné (maeliss.peigne@inserm.fr)

25 **Structured Abstract**

26 **Study question:** Does the relative distribution of AMH isoforms differ from one patient to
27 another depending on their BMI and PCOS status in the serum and the follicular fluid (FF)?

28 **Design:** Obese and normal weight (NW) patients (PCOS:70; non-PCOS:37) were selected
29 from a database (2014-1018) for this case-control study in the serum. Between 2018 and
30 2019, obese (N = 19) and NW(N = 20) women with or without PCOS, receiving IVF
31 treatment, were included in the FF study. The bio-banked serums and FF were tested for
32 totalAMH (proAMH and AMH_{N,C} combined) and proAMH using an automatic analyzer. The
33 AMH prohormone index (API= [proAMH]/[total AMH]x100) was calculated as an inverse
34 marker of conversion of proAMH to AMH_{N,C}, with only the latter isoform that could bind to
35 the AMH receptor-complex.

36 **Results:** API was not significantly different between controls and women with PCOS.
37 However, obese women versus their NW counterparts had a lower API. Grouping PCOS and
38 controls, a lower API was found in obese compared to NW women (34.9% vs 41.2%; $P <$
39 0.001), suggesting a greater conversion of proAMH to AMH_{N,C}. The API in the serum was
40 significantly correlated with metabolic parameters. In the FF, API is not different between
41 obese and NW women independently of PCOS (67.4% vs 71.1%; $P = 0.5$) and is higher than
42 in the concomitant serum (32.8% in obese and 40.4 % in NW women).

43 **Conclusions:** The proportion of inactive form of AMH in the serum is higher in NW versus
44 obese women but not in the FF, independently of PCOS. The conversion of proAMH into the
45 cleaved isoform is likely occurring in extra-ovarian tissues and exacerbated in obese
46 individuals.

47

48 **Key Message**

49 A lower proportion of proAMH is detectable in the serum of obese compared with normal-
50 weight women independently of PCOS status. In the follicular fluid this difference is not
51 found suggesting that a greater cleavage of proAMH to AMH_{N,C} is likely occurring in extra-
52 ovarian tissues and exacerbated in obese as compared to normal weight women.

53

54 **Keywords:** PCOS; AMH; molecular forms; BMI; obesity; follicular fluid

55 **Introduction**

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

62 A frequent hallmark found in women with PCOS, is an elevation of Anti-Müllerian hormone
63 (AMH) serum levels, which have been reported to be 2-3-fold higher than in women with
64 healthy ovaries (Cook et al., 2002; Pigny et al., 2003). Interestingly, the severity of the PCOS
65 phenotype correlates with AMH production, which is higher in anovulatory than in ovulatory
66 PCOS patients (Abbara et al., 2019; Catteau-Jonard et al., 2012; Pellatt et al., 2007).
67 Clinically observed correlations between enhanced basal levels of AMH and PCOS have led
68 to a potential physiological role for AMH in “follicular arrest” in women with PCOS
69 (Dewailly et al., 2014). In this pathology, enhanced numbers of primary and small secondary
70 follicles secrete abnormally high levels of AMH, that associates with excess androgen
71 production/secretion and leading to arrested follicular maturation.

72 AMH is a glycoprotein synthesized as a homo-dimeric prohormone of 140 kDa (composed of
73 two monomers of 70 kDa), which undergoes a proteolytic cleavage at monobasic sites to
74 become biologically active (di Clemente et al., 2010; Pepinsky et al., 1988). The cleavage of
75 AMH gives rise to a 110 kDa N-terminal homodimer formed by two 57 kDa subunits
76 (AMH_N) and a 25 kDa active C-homodimer composed of two identical 12.5 kDa (AMH_C)
77 subunits. These 2 homodimers remain associated in a non-covalent complex (AMH_{N,C})

78 (Nachtigal and Ingraham, 1996; Pepinsky et al., 1988). Although having no intrinsic activity,
79 AMH_N has the role of amplifying the biological activity of the AMH_C (Wilson et al., 1993).
80 AMH_{N,C} and its pro-hormone (proAMH) are found in blood but there is little evidence for the
81 presence of free AMH_C (Pankhurst and McLennan, 2013). This suggests that circulating
82 cleaved AMH_{N,C} levels represent the receptor-competent isoform available for a hormonal
83 function in a variety of tissues and organs expressing the exclusive binding- receptor AMHR2
84 (Barbotin et al., 2019).

85 A number of studies point to AMH as a useful marker for the diagnosis of PCOS, however
86 challenges to the use of AMH to diagnose PCOS have been fully reviewed in Teede et al.
87 2018 (Teede et al., 2018). A recent study by Abbara and colleagues (Abbara et al., 2019) has
88 suggested that AMH has potential as a biomarker for diagnosis of PCOS, outperforming
89 antral follicles count, and greater elevations in AMH were associated with a more certain
90 diagnosis of PCOS. However, due to the variability in levels using first-generation and less
91 reliable assays and the lack of an international standard, AMH has not yet been adopted as
92 part of the diagnostic criteria for PCOS (Teede et al., 2018).

93 In recent years, studies have started to use novel approaches and analytical assays (Pankhurst
94 et al., 2017; Wissing et al., 2019) aiming at determining whether the ratios of proAMH or
95 AMH_{N,C} in serum are altered in women with PCOS relative to women without PCOS and to
96 understand if different AMH-related isoforms can be used to predict PCOS. However, the
97 results of those studies are conflicting, either reporting differences in the mean
98 proAMH:AMH_{N,C} ratio in the PCOS group versus the unaffected control group (Pankhurst et
99 al., 2017) or reporting no differences in the relative distribution of the AMH isoforms
100 between those groups (Wissing et al., 2019). We hypothesized that part of the discrepancies
101 could be due to the heterogeneity of the disorder and that stratifying women with and without

102 PCOS based on their metabolic phenotype (body mass index) could better help to pin-point
103 eventual differences in AMH cleavage. These difference in AMH cleavage could lead to
104 relative higher levels of active AMH in the serum which could participate to the existence of
105 different PCOS phenotypes.

106 The aims of this study were thus to determine if the relative distribution of AMH isoforms
107 may differ from one patient to another depending on their body mass index (BMI) and PCOS
108 status and to investigate whether differences in the proportions of AMH isoforms are due to
109 increased proAMH cleavage occurring in the follicular fluid or in the serum.

110

111 **Materials and Methods**

112 *Population for early follicular phase serum study*

113 From a database including clinical, hormonal and ultrasound features that were consecutively
114 recorded between January 2014 and December 2018 in patients referred to our department in
115 an academic hospital for hyperandrogenia (HA) and/or oligo-anovulation and/or infertility. 70
116 women with PCOS (42 obese; BMI ≥ 30 kg/m², and 28 normal weight; BMI ≤ 25 kg/m²) and
117 37 controls (16 obese and 21 normal weight) were selected retrospectively, as described
118 below. Patients were included in the PCOS group according to a modified Rotterdam
119 classification (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group,
120 2004), i.e., presence of at least 2 out of the 3 following items: -1) HA (clinical or biological;
121 as previously described (Peigné et al., 2018); -2) oligo-anovulation, (i.e. oligomenorrhea or
122 amenorrhea); -3) presence of Polycystic Ovarian Morphology (PCOM) at Ultrasound (U/S),
123 with an ovarian area ≥ 5.5 cm² and/or a follicle number per ovary ≥ 19 , according to our in-
124 house thresholds, as previously reported (Dewailly et al., 2011) .

125 Controls were referred to our department for infertility due to tubal and/or sperm abnormality.
126 None of these women had any component of the Rotterdam classification: they had regular
127 cycles, no hyperandrogenism and normal ovaries at U/S.

128 All medications known to affect metabolism (e.g. metformin) or ovarian function (e.g. oral
129 contraceptive pill) had to be stopped at least 3 months before blood tests and U/S.

130 *Investigations*

131 During the medical examination, age, height, weight and waist circumferences were
132 registered. Blood sampling was performed in the early follicular phase (i.e. between cycle
133 days 2 and 5; either spontaneous or induced by didrogestrone). Follicle Stimulating Hormone
134 (FSH), Estradiol (E2), Luteinizing Hormone (LH), Total Testosterone (TT), Fasting Serum
135 Insulin levels (I) were measured by immunoassay using an automatic analyzer (Architect,
136 Abbott Laboratories, USA). Sex Hormone Binding Globulin (SHBG) levels were measured
137 by immunoassay using the Immulite analyzer (Siemens, Germany). Androstenedione levels
138 were determined by LC-MS-MS.

139 Biochemical tests (glucose, lipid levels) were performed in the central biochemistry
140 department of the hospital using routine assays on Roche analyzers.

141 *Population for follicular fluid and concomitant serum study*

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

148 *Follicular fluid samples*

149 During the oocyte pick-up procedure, all follicles over 12 mm were retrieved. After oocyte
150 isolation for IVF procedure, follicular fluid was collected, centrifuged at 2,000 rpm for 5 min
151 to remove cells and frozen at -80° until analysis.

152 *AMH measurements*

153 Routinely, fully automated Access Dxi sandwich chemoluminescent immunoassays
154 (Beckman Coulter) were used to measure serum AMH levels. The standard used in the Access
155 Dxi assay is the human recombinant 140 kDa AMH. Its limit of detection is around 0.47
156 pmol/L (Pigny, unpublished) and its measurement range is 0.47-150 pmol/L (van Helden and
157 Weiskirchen, 2015). The Access Dxi assay used the same antibodies pair than the AMH Gen
158 II ELISA: a capture antibody binding C-term part of AMH part and a detection antibody
159 initially thought to be directed against the C-term of AMH but which also required the N-
160 terminal part of AMH for correct detection (Kevenaar et al., 2006; Pankhurst et al., 2014).
161 Based on these features, it can be assumed that the Access Dxi assay recognizes in the
162 biological samples both the pro-AMH (uncleaved) and the AMH_{N,C} (receptor competent-
163 active). To fix this point, four serums were spiked with 10 to 25 ng/ml of recombinant AMH_C
164 (25 kDa, R& D Systems, MIS 1737). Results showed the absence of cross-reactivity of
165 AMH_C in this assay (**Supplementary Table 1**). So we concluded that the only molecular
166 AMH forms detected in the serum by Dxi Automatic analyzer were proAMH + AMH_{N,C} that
167 we defined as total AMH (**Supplementary Figure 1A**).

168 *Specific measurement of AMH molecular forms in serum and follicular fluid*

169 A specific assay for proAMH measurement was built for Beckman Coulter Dxi automatic
170 analyzer based on previous protocol designed with the AMH Gen II ELISA (Pankhurst and

171 McLennan, 2016) and used in the left-over serum or follicular fluid of the patients. This
172 experimental protocol included a deoxycholate (DOC) treatment step of the serum which
173 leads to the dissociation of the non-covalent link between N-terminal and C-terminal parts of
174 AMH_{N,C} which prevents binding of the detection antibody. ProAMH is unaffected by the
175 DOC treatment as the molecule is uncleaved (**Supplementary Figure 1B**).

176 With the Gen II ELISA the DOC treatment step was performed between the capture and
177 detection steps which is feasible in a manual assay but not possible with an automatic assay.
178 Therefore, we decided to pre-treat the samples before assaying AMH with the analyzer. Based
179 on Pankhurst's study (Pankhurst and McLennan, 2016), we performed independent
180 experiments to set up DOC concentration, incubation time and temperature. The final protocol
181 is described hereafter.

182 Two 150 µL aliquots were made from each sample (bio-banked serum or follicular fluid,
183 stored at -80°). Phosphate Buffer Saline (0.02 M, pH = 6.8, 37°C) was added in one aliquot
184 (control) whereas Sodium Deoxycholate in PBS buffer at 37°C (final concentration 0.23%,
185 i.e. 2333 µg/ml, corresponding to nearly 6000 times more than the endogenous serum DOC
186 concentration, was added in the second aliquot. Aliquots were incubated at 37°C during 15
187 min before being spiked again with DOC or PBS solution and further incubated for 15 min at
188 37°C. Final volumes of both aliquots were 300 µL which allow a direct measurement with the
189 Beckman Coulter Access Dxi automatic analyzer. Control aliquot with PBS allows the
190 measurement of total AMH in the sample whereas aliquot with DOC treatment allows the
191 measurement of proAMH only (**Supplementary Figure 1**).

192 The AMH prohormone index (API; (Pankhurst and McLennan, 2016)) was calculated as a
193 surrogate marker of conversion of proAMH to AMH_{N,C} ; $API = \frac{[proAMH]}{[total AMH]} \times$
194 100.

195 *Validation of the specific proAMH assay*

196 No difference in total AMH levels measured with the Dxi Analyzer was observed between
197 proAMH (either standard of the Access Dxi assay or rhAMH provided by Dr. Michael
198 Pankhurst (Pankhurst and McLennan, 2016)) treated by DOC versus PBS buffer
199 (**Supplementary Table 2**) thus confirming that proAMH is unaffected by the DOC treatment.

200 As previously described (Pankhurst and McLennan, 2013), an aliquot of rhAMH was treated
201 with furin to convert proAMH to AMH_{N,C}. By western blotting we observed that the untreated
202 preparation (rhAMH) mainly contains proAMH (around 77% after densitometric analysis)
203 whereas the furin-treated preparation mainly contains AMH_{N,C} (around 60%)
204 (**Supplementary figure 2**). Both preparations were measured after dilution using our specific
205 assay for proAMH. As expected, addition of DOC induced a strong decrease of the AMH
206 concentration in the furin -treated preparation. This demonstrated that DOC treatment
207 reduces the assayed AMH value in a manner that is consistent with dissociation of the
208 AMH_{N,C} complex. (**Supplementary figure 2**). The assayed AMH values after DOC
209 pretreatment were not modified by the lipid profile of the dilution medium (serum of
210 menopausal women with or without dyslipidemia, or in the standard of the Access Dxi assay,
211 which does not contain AMH nor lipids) (**Supplementary figure 2**).

212 *Statistical analysis*

213 All statistical analyses were performed using SPSS software 22.0 (SPSS Inc., Chicago, IL,
214 USA). Results were expressed as medians, with 5^e-95^e or 10^e -90^e percentiles for continuous

215 variables and by the frequencies and percentages for categorical variables. Comparisons
216 between two independent groups were performed using the Mann-Whitney U test and
217 between dependent groups (for paired comparison between follicular fluid and serum results)
218 using the Wilcoxon signed rank test. Comparisons between four groups were performed using
219 the ANOVA test with post-hoc pairwise comparisons between obese and normal weight
220 controls, and obese and normal weight PCOS after applying Bonferroni correction. The
221 correlations between the various parameters were expressed as Spearman's correlation
222 coefficient (r). A p value of <0.05 was considered to be significant and a r absolute value \geq
223 0.4 was considered to be strong and clinically relevant.

224 ***Ethics***

225 For the first part of the study, all patients had previously given their informed consent for the
226 use of their clinical, hormonal, ultrasound records and leftover serum. For the second part of
227 the study, all patients gave their informed consent for the use of their follicular fluid and
228 leftover serum. The Institutional Review Board of the University Hospital gave its
229 unrestricted approval to the anonymous use of clinical, hormonal, ultrasound records and
230 biologic collection from all patients (reference DC-2008-642).

231

232 **Results**

233 ***Molecular forms of AMH in early follicular phase serum***

234 One hundred-seven women were selected for this first part of the study: 70 women with
235 PCOS (42 obese and 28 normal weight) and 37 controls (16 obese and 21 normal weight).
236 **Table 1** shows the main clinical, hormonal, metabolic and U/S characteristics of the study
237 population.

238 Mean total AMH and proAMH levels were higher in women with PCOS, independently of
239 their metabolic features (BMI and insulin resistance, indirectly evaluated by waist
240 circumference, SHBG levels and fasting insulinemia), when compared with controls
241 (respectively for total AMH: PCOS: 47.4pmol/L IC₅₋₉₅ (26.5 – 133.5) versus
242 controls:17.2pmol/L IC₅₋₉₅ (7.8 – 35.7); $P < 0.001$ and proAMH: PCOS: 19.6pmol/L IC₅₋₉₅
243 (7.5 – 55.3) versus controls: 6.6 pmol/L IC₅₋₉₅ (2.6 – 15.9); $P < 0.001$).

244 The AMH prohormone index (API = [proAMH]/[total AMH] x 100) was then used as a
245 surrogate marker of conversion of proAMH to AMH_{N,C} (i.e: low index indicating increased
246 proAMH conversion into receptor-competent isoform). No difference in the API was found
247 between patients with and without PCOS when we stratified the population study group based
248 only on PCOS diagnosis (PCOS: 34.6% IC₅₋₉₅ (28.6 - 48.3) versus controls: 38.2% IC₅₋₉₅
249 (28.7 – 51.0); $P = 0.8$). However, when we split by BMI, we observed that obese women
250 versus their normal weight counterparts in both control and PCOS groups had an API
251 significantly lower, suggesting a greater conversion of proAMH to AMH_{N,C} (**Figure 1**).
252 Serum total AMH and proAMH levels were not significantly different between obese and
253 normal weight women in each group (**Figure 1**).

254 Possible interactions between the API and pathophysiological features of PCOS were then
255 investigated. When we grouped PCOS patients and controls, a significantly lower API was
256 found in obese compared to normal weight women (34.9 % vs 41.2% respectively; $P < 0.001$),
257 whereas total AMH and proAMH levels were not significantly different.

258 In the whole population (N = 107), the API negatively correlated to metabolic parameters:
259 weight ($r = -0.578$), BMI ($r = -0.557$), waist circumference ($r = -0.530$), Triglycerides level (r
260 = -0.191) and fasting insulinemia ($r = -0.351$; **Figure 2, Table 2**). Moreover, the API was
261 found to be positively correlated with SHBG ($r = 0.293$), and HDL cholesterol level ($r =$

262 0.221) (**Figure 2, Table 2**) No significant correlation was found with serum androgens or LH
263 levels (**Table 2**).

264

265 *Molecular forms of AMH in follicular fluid and serum of patients at the end of ovarian*
266 *stimulation*

267 ProAMH is known to be secreted by granulosa cells and thus released into the follicular fluid
268 before reaching the serum (Campbell et al., 2012). We next tried to determine whether the
269 cleavage of this protein occurs within the ovaries or in the serum by analysing the API in
270 those samples.

271 Because the API in early follicular phase serum was only different between obese and normal
272 weight women, this second part of the study focused on thirty-nine patients: 20 normal weight
273 women (including 10 with PCOS) and 19 obese women (including 9 with PCOS). The
274 proportion of patients with PCOS in both groups (obese and normal weight) was not different.
275 The main clinical, hormonal, metabolic, U/S and ovarian stimulation features of this second
276 study population are shown in **Supplementary Table 3**.

277 We measured total AMH and proAMH in the follicular fluid (FF) retrieved after oocyte pick-
278 up as well as in the concomitant sampled serum of each woman. The total AMH and the
279 proAMH were comparable in normal weight versus obese women when comparing the
280 protein amount either in the FF or in the serum (**Figure 3**); respectively in the FF: for total
281 AMH 18.9 pmol/L IC₅₋₉₅ (7.2-56.4) vs. 21.4 pmol/L IC₅₋₉₅ (6.6-86) and for proAMH 13.1
282 pmol/L IC₅₋₉₅ (4.9-43.4) vs. 15.4 pmol/L IC₅₋₉₅ (3.6-76). When we analysed the API, we found
283 no significant differences in the FF between the two study groups, while the API was
284 significantly reduced in the concomitant serum of obese women as compared to normal

285 weight women (**Figure 3**). Interestingly, in both normal weight and obese women, the API
286 was significantly higher in the follicular fluid than in matched serum samples (**Figure 3**), thus
287 suggesting that most of the cleavage of proAMH occurs in the general circulation rather than
288 in the ovaries.

289 Lastly, no correlation was found between the proAMH ratio in follicular fluid and any
290 clinical, hormonal, metabolic, ultrasonographic or ovarian stimulation parameters studied
291 parameter (**Table 2**; univariate analysis in the whole population, N = 39).

292

293 **Discussion**

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

305 Our data confirm and expand a recent study showing that the ratio of AMH isoforms do not
306 differ between PCOS and control subjects (Wissing et al., 2019), thus implying a similar
307 distribution of uncleaved (inactive) and cleaved AMH in the two study groups. Wissing and

308 colleagues analysed a study group composed by women with either a normal weight, slightly
309 overweight and/or comprising some PCOS women that received metformin and they reported
310 a negative correlation between metabolic parameters and the proportion of inactive AMH
311 form in the serum. Here, we confirmed the correlation results found in Wissing et al., with an
312 even greater coefficient of correlation between the API and BMI ($r = - 0.557$) and waist
313 circumference ($r=-0.530$) and demonstrated that a greater processing of AMH in the
314 circulation does not associate with PCOS but rather with a severe metabolic disturbance such
315 as obesity.

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

325 Our data suggest that the cleavage of proAMH is only partly occurring at the follicular level
326 and that most of the cleavage of proAMH is mainly occurring in extra-ovarian tissues.

327 Subtilisin/kexin-like proprotein convertases such as furin (PCSK3) or PCSK5 are thought to
328 be responsible for AMH cleavage (Nachtigal and Ingraham, 1996). Plasmin is also a
329 candidate for proAMH cleavage because, *in vitro*, recombinant proAMH can be cleaved by
330 plasmin (Pepinsky et al., 1988). Some authors described that proAMH cleaving proteases are

331 found in vascular tissues (reviewed in (McLennan and Pankhurst, 2015). However, the *in vivo*
332 cleavage of proAMH remains largely unknown and it likely occurs at several levels/tissues.
333 Interestingly, serum furin levels are known to be modulated by inflammatory conditions such
334 as that one that can be found in metabolic or cardio-vascular diseases (Fathy et al., 2015; Ren
335 et al., 2017). Further studies are required to assess the *in vivo* molecular nature of the AMH
336 cleavage, the exact anatomical sites of AMH conversion and whether this could be influenced
337 by the metabolic status of the individual.

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

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

354 In conclusion, this study showed that circulating AMH isoform concentrations cannot be used
355 to improve the precision of PCOS phenotype diagnosis. However, the novelty of this study
356 relies on the findings showing that: 1) a higher proportion of cleaved, most likely bioactive,
357 AMH (AMH_{N,C}) is detectable in the serum of obese women compared to women with normal
358 weight and 2) a greater cleavage of proAMH to AMH_{N,C} is likely occurring in extra-ovarian
359 tissues and exacerbated in obese women. This study opens new avenues of investigation
360 aimed at addressing novel roles of AMH in the context of metabolic disturbances associated
361 with reproductive alterations.

362

363 **Acknowledgments**

364 We would like to thank Dr Isabelle Loison, Dr Anne -Laure Barbotin, Dr Virgine Simon,
365 Lydie Lombardo and Sylvie Vanoverschele for their precious help in collecting samples.

366

367

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528

529 **Fundings**

530 This work was supported by the Institut National de la Santé et de la Recherche Médicale
531 (INSERM), France [grant number U1172], by the European Research Council (ERC) under
532 the European Union's Horizon 2020 research and innovation program (ERC-2016-CoG to
533 P.G. grant agreement n° 725149/REPRODAMH), by the Centre Hospitalier Régional
534 Universitaire, CHU de Lille, France (Bonus H to P.G.).

535

536 **Conflict of interest**

537 The authors have no conflict of interest to declare.

538

539 **Vitae**

540 Maëliiss Peigné is a Gynecologist sub-specialized in Reproductive Medicine working at AP-
541 HP-Université Sorbonne Paris Nord, Bondy, France and a PhD student in INSERM U1172,
542 Lille, France. Her main research interests include Polycystic Ovary Syndrome
543 pathophysiology and management, fertility preservation and improvement of ART results.

544

545

546 **Figure legends**

547

548 **Figure 1: AMH molecular forms in the early follicular phase (EFP) serum: women with**
 549 **polycystic ovary syndrome (PCOS) versus controls split by body mass index (BMI).**

550 Total AMH (pmol/L), AMH proprotein (proAMH) (pmol/L) and API = (proAMH/totalAMH)
 551 x100 in women with PCOS and controls, according to their BMI (obese = BMI \geq 30.0 kg/m²)
 552 and normal weight (BMI \leq 25.0 kg/m). *Green dots*, control women (N = 37); *darker green*
 553 *dots*, obese control women (N = 16); *orange dots*, women with PCOS (N = 70); *darker*
 554 *orange dots*, obese women with PCOS (N = 42). Data are presented as medians with
 555 interquartile ranges. **** $p < 0.001$** ; a: different from normal weight control (NC); b: different
 556 from obese control (OC); c: different from normal weight women with PCOS (NP); d:
 557 different from obese women with PCOS (OP). Groups that are not linked by a line did not
 558 exhibit statistically significant differences.

559

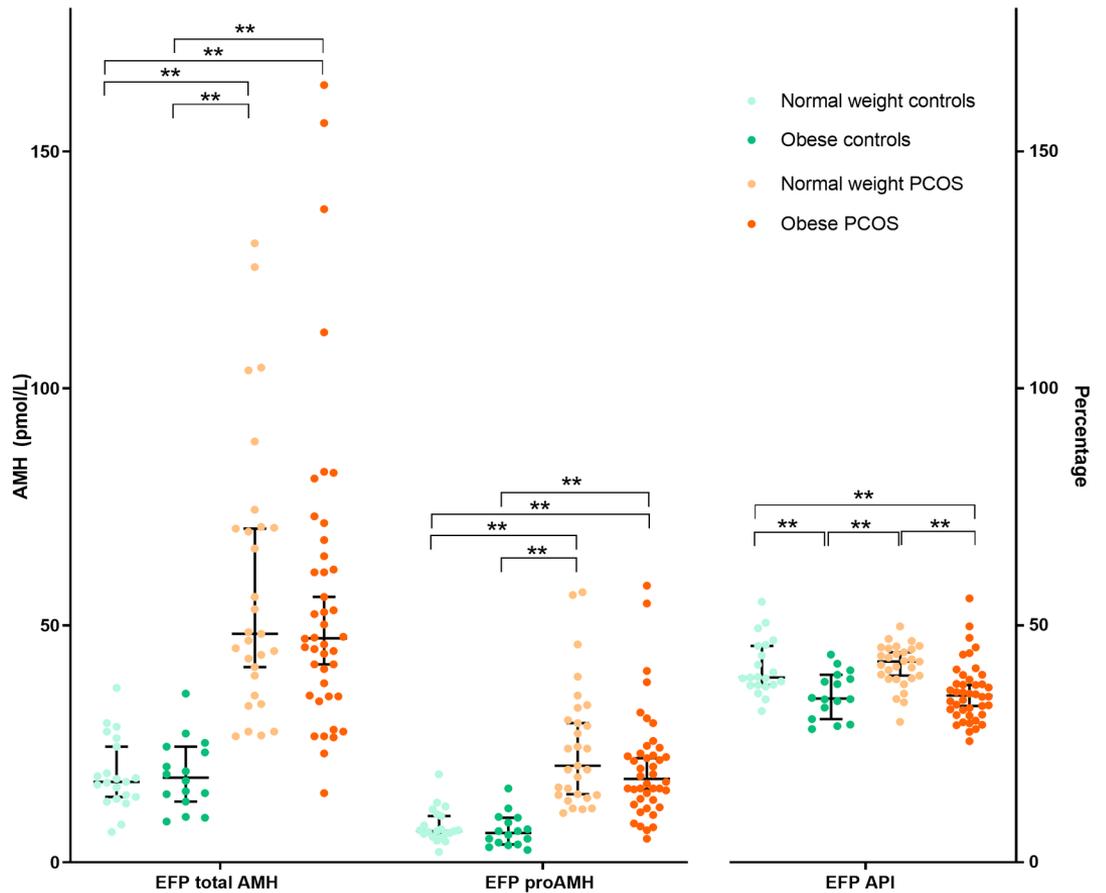
560 **Figure 2: Correlation of AMH prohormone index (API) in the early follicular phase**
 561 **serum with metabolic parameters.** Spearman correlation; EFP: early follicular phase, BMI:
 562 body mass index; SHBG: Sex Hormone Binding Globulin; HDL: High density lipoprotein;
 563 API: (proAMH/totalAMH)x100 , **** $p < 0.001$** , r = correlation coefficient.

564

565 **Figure 3: Proportion of AMH molecular forms in the follicular fluid (FF) and in the end**
 566 **of ovarian stimulation serum (Serum): normal weight versus obese women.** API (API:
 567 AMH prohormone index (proAMH/totalAMH) x 100) values in follicular fluid (FF) (on the
 568 left) and serum at the end of ovarian stimulation for IVF (on the right) of the same obese or
 569 normal weight women. *Light blue*, normal weight women (n = 20); *dark blue*, obese women

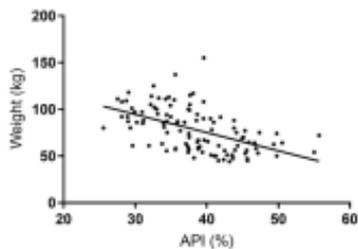
570 (n = 19); *triangles*, follicular fluid (on the left); *circles*, serum (on the right). Data are
571 presented as medians with interquartile ranges. $**p < 0.001$.

572

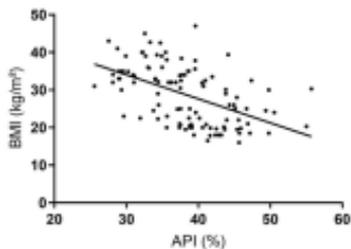


	Control (C)		PCOS (P)	
	Normal weight (NC) Med (5e-95e) N= 21	Obese (OC) Med (5e-90e) N= 16	Normal weight (NP) Med (5e-95e) N= 28	Obese (OP) Med (5e-95e) N= 42
EFP total AMH (pmol/L)	17 (6.56 - 36.06) c,d	17.9 (8.6 - 29.72) c,d	48.4 (26.7 - 128.3) a,b	47.3 (23.51 - 153.3) a,b
EFP pro AMH (pmol/L)	6.6 (2.42 - 18.0) c,d	6.2 (2.6 - 12.66) c,d	22.2 (10.8 - 56.7) a,b	17.6 (6.9 - 52.5) a,b
EFP API (%)	39.02 (32.2 - 54.5) b,d	34.6 (28.12 - 42.48) a,c	42.4 (31.5 - 48.6) b,d	35.22 (27.63 - 49.42) a,c

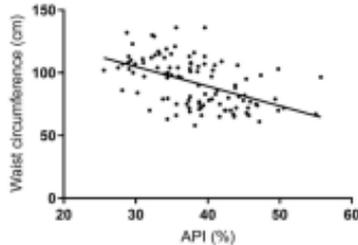
$r = -0.578^{**}$



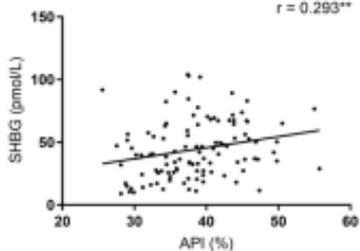
$r = -0.557^{**}$



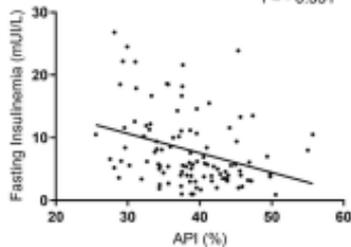
$r = -0.530^{**}$



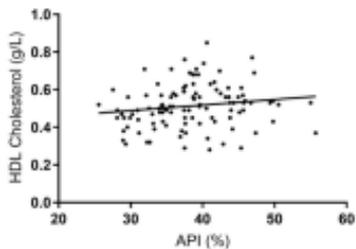
$r = 0.293^{**}$



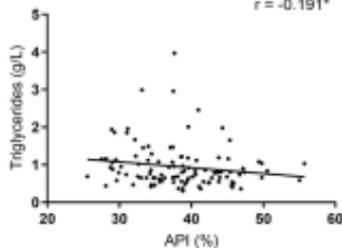
$r = -0.351^{**}$

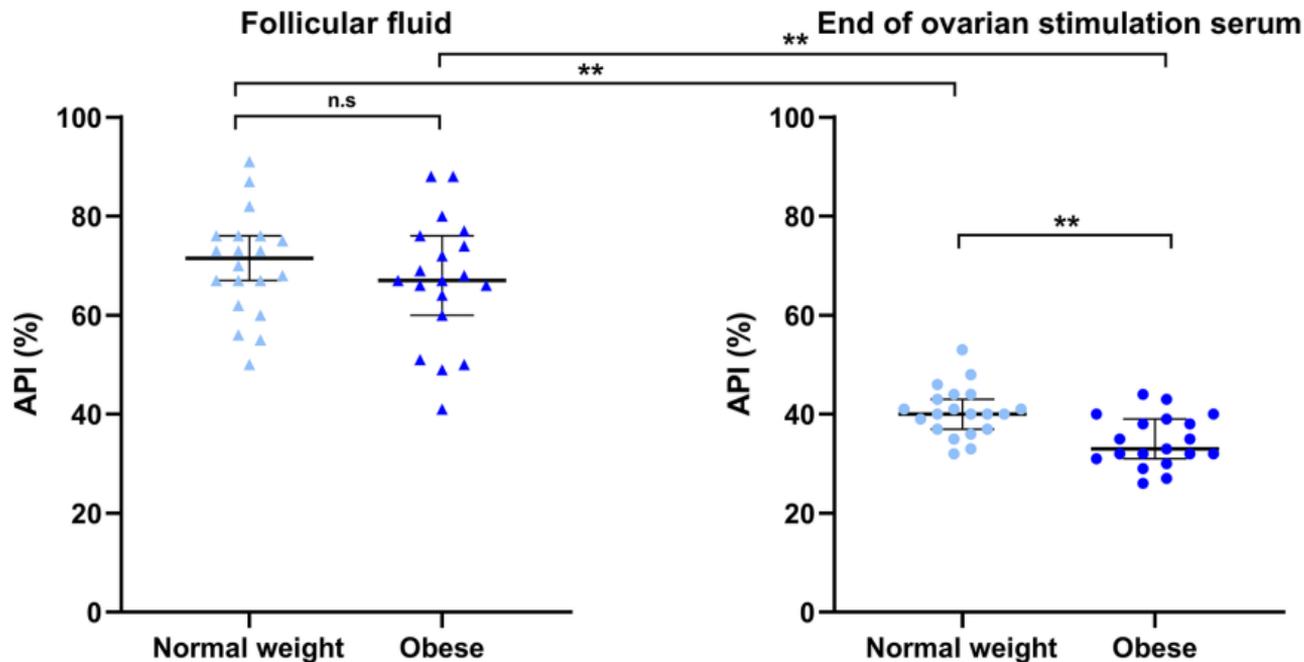


$r = 0.221^*$



$r = -0.191^*$





	Normal weight N = 20 Med (10e-90e)	Obese N = 19 Med (10e-90e)	<i>P</i>
FF total AMH (pmol/L)	18.9 (7.2 – 56.4)	21.4 (6.6 – 86)	0.627
FF pro AMH (pmol/L)	13.1 (4.9 – 43.4)	15.4 (3.6 – 76)	0.879
FF API (%)	71.1 (55.0 – 86.8)	67.4 (48.6 – 88.2)	0.478
Serum total AMH (pmol/L)	12.9 (4.4 – 44.7)	10.4 (3.0-70.2)	0.444
Serum pro AMH (pmol/L)	5.2 (1.8 – 18.2)	3.6 (1.2-22.2)	0.224
Serum API (%)	40.4 (33.0-48.1)	32.8 (26.9-43.2)	0.001

Table 1: Population characteristics in the early follicular phase serum study

	Control (N=37)		PCOS (N=70)	
	Normal weight (NC) N= 21 Med (5-95)	Obese (OC) N= 16 Med (5-90)	Normal weight (NP) N= 28 Med (5-95)	Obese (OP) N= 42 Med (5-95)
Age (y)	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
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
BMI (kg/m²)	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
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
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
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
Estradiol (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)
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
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)
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
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
Fasting Insulinemia (mUI/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
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)
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
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)
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
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

a: different from NC; b: different from OC; c: different from NP; d: different from OP ; PCOS: polycystic ovary syndrome; BMI: body mass index; SHBG: Sex Hormone Binding Globulin; LH: luteinizing hormone; FSH: Follicle stimulating hormone; HDL: High density lipoprotein ; LDL : Low density lipoprotein; AFC: antral follicular count

Table 2: Correlation of proAMH ratio in the serum and follicular fluid with clinical, hormonal, ultrasonographic, and metabolic parameters

	Serum EFP API <i>rho Spearman</i>	FF API <i>rho Spearman</i>
Age	-0,16	,088
Weight	-,578**	-,192
BMI	-,557**	-,094
Waist Circumference	-,530**	-,262
Testosterone	-0,168	-,052
Androstendione	-0,073	-,080
Estradiol	-0,025	,092
LH	0,042	-,128
FSH	0,134	,278
SHBG	,293**	,650
Fasting Glycaemia	-0,121	-,285
Fasting Insulinemia	-,351**	-,550
Total cholesterol	-0,056	
HDL Cholesterol	,221*	
LDL cholesterol	-0,092	
Triglycerides	-,191*	
Total AFC both ovaries	0,003	-,062
Serum total AMH at the end of stimulation (ES)		-,197
Serum proAMH at ES		-,136
Serum pro AMH ratio at ES		,283

Spearman correlations ($\pm 0,400 \Rightarrow$ clinically relevant), EPF: early follicular phase, FF: follicular fluid; BMI: body mass index; SHBG: Sex Hormone Binding Globulin; LH: luteinizing hormone; FSH: Follicle stimulating hormone; HDL: High density lipoprotein; LDL : Low density lipoprotein; AFC: antral follicular count, API: AMH prohormone index, *p<0.05, ** p<0.01