Article

Determination of Fertility Hormones and Adipokines by LC-MRM/MS Analysis

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ABSTRACT: Due to the increase in the rate of male and female infertility, assisted fertilization practices are currently adopted as valid support for couples unable to get pregnant. Analytical approaches for fertility hormone dosages are constantly being developed, following the technological progress of fertilization methods that have evolved for more than a century. Indeed, the analysis of fertility hormones in serum samples is a common clinical practice to check the fertility state, but absolute quantification of these hormones is a great challenge due to biological variability and low serum concentrations. Currently, ELISA (enzyme-linked immunosorbent assay) based methods are the most used analytical techniques to quantify hormones in blood in clinical settings. The current Article discusses the development of a liquid chromatography-tandem mass spectrometry method (LC-MS/MS) to monitor multiple fertility hormones of a protein nature in a single chromatographic run, i.e., LH (luteinizing hormone), FSH (follicle-stimulating hormone), TSH (thyroid-stimulating hormone), AMH (anti-Müllerian hormone), adiponectin, ghrelin, leptin, glucagon, and obestatin. Particular attention has been paid to the AMH hormone, whose ELISA-based quantification is known to be controversial due to the poor reproducibility between the various kits used. For AMH, the internal standard method was used for the quantitative determination to compare mass spectrometry data to the ELISA assays performed by an accredited analysis laboratory on a cohort of samples from women aged between 18 and 60 years. The ability to monitor multiple transitions by LC-MRM/MS ensured both high specificity and high selectivity, which is necessary for the quantification of protein and steroid hormones, besides improvements in data reproducibility and reduced analysis times and costs.
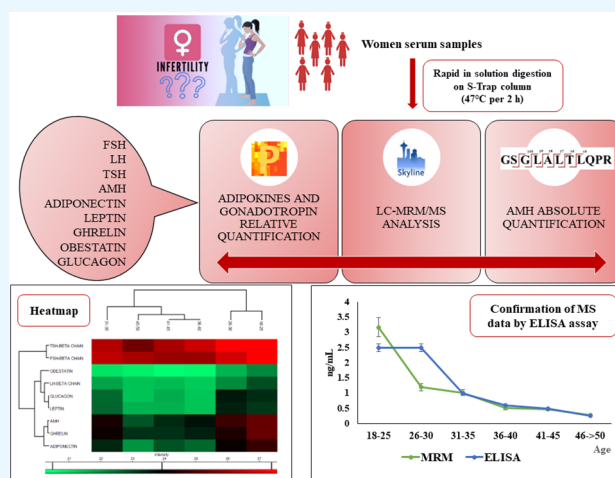
1. INTRODUCTION

Infertility, defined as one year of attempted conception without success, is one of the most prevalent chronic health disorders involving young adults.[1−4] Data from population-based studies suggest that 10−15% of couples around the world experience infertility. Causes of infertility in females include ovulation problems, weight changes, hormone imbalance, and age-related issues.[3,5]

Advancing age naturally affects the state of fertility, mainly due to the reduction of the ovarian reserve. Indeed, ovarian quality is reported to decline up to 81% exclusively due to age, with reductions to 12% at age 30 and 3% at the age of 40, making this factor one of the most crucial in female infertility. The reduction of hormonal levels is also strictly correlated with the success of achieving a pregnancy, suggesting the need to warn against delaying motherhood beyond 35 years old.[3,5] Numerous hormones, classified as proteins or steroids, are involved in reproduction and fertility, and their concentrations vary according to gender and age. Hormonal imbalance is a major cause of anovulation. It has been established that women with a hormonal imbalance do not produce enough oocytes to ensure the development of an egg.[4,6] In this context, clinical analysis of fertility hormones in blood samples is a common clinical practice and is currently crucial for assessing the health status and level of fertility in women. The most used analytical technique to quantify protein fertility hormones is ELISA (enzyme-linked immunosorbent assay), an extremely sensitive method based on the recognition of antigens using a specific antibody. In the current Article, we propose an alternative

method to immunoassays suffering from high antibody cross-reactions, especially in multiplex assays. Such a method is based on tandem mass spectrometry in the multiple reaction monitoring (MRM) ion mode to quantify a wide panel of hormonal proteins, including gonadotropins, TSH (thyroid-stimulating hormone), adipokines, and AMH (anti-Müllerian hormone), in a single chromatographic run. The use of LC-MRM/MS in diagnostics is already well-known and consolidated for both proteomics[4,7−10] and metabolomics.[11−14] In a previously published study by Illiano et al.,[4] a method was developed for the quantification of FSH (follicle-stimulating hormone), LH (luteinizing hormone), and TSH in serum samples from women proposed for clinical investigation or routine analysis. Hence, the expansion of the protein panel to be monitored through the inclusion of adipokines, which are recognized to be equally crucial in fertility. Finally, particular emphasis has been also given to the quantification of the AMH protein, whose dosage is controversial in the literature due to data poor reproducibility between the various kits used for the analysis.[15,16] Mass spectrometry proved to be a valid approach to determining the AMH dosage even when comparing the data with ELISA assays performed by an accredited analysis laboratory for a cohort of samples from women aged between 18 and 60 years.

Exploiting the multiplexed monitoring of several protein hormones by LC-MRM/MS allowed to increase the analysis selectivity, offering the improvement of analytical robustness and the reduction of analysis time and cost.

2. RESULTS AND DISCUSSION

The serum dosage of gonadotropins, TSH, and AMH concentrations by immunoassay is a routine practice in clinical applications in the diagnosis of ovarian conditions and for predicting the response to *in vitro* fertilization (IVF) therapy protocols.[17,18] Currently, the mass spectrometry platform is becoming a valid support to clinical laboratories, especially in metabolomics, as the sample manipulation is minimal and the analysis cost and time are enormously decreased using a targeted approach by LC-MRM/MS. The proteomics approach suffers from the digestion step, which lasts around 16 h for enzymatic hydrolysis. In the current study, we proposed an optimized protocol for digestion on S-Trap columns that allowed to reduce the analysis time to 2 h of reaction to meet the requests of clinical laboratories to release a report within 24 h. Furthermore, the objective of this Article is the development of a method orthogonal to the immunological assays for the quantification of a wide panel of nine total proteins, *i.e.*, three gonadotropins (FSH, LH, and AMH), a thyroid hormone (TSH), and five adipokines (adiponectin, leptin, glucagon, obestatin, and ghrelin) to be monitored in a single chromatographic run by using a targeted mass spectrometry approach. The present Article mainly deals with the validation of the method for AMH quantification by using heavy internal standard peptides and the subsequent implementation of the MRM/MS method by comparing data with those obtained by ELISA assays performed by the accredited clinical laboratory.

2.1. AMH: Method Validation by Internal Standard Method. Isotopically labeled AMH (209−219) peptide was used to develop the quantitative analysis method. Table S1 reports the monitored transitions selected for each heavy and light peptide. These peptides were used to create the in-matrix

calibration curves (according to section 4) using a concentration ranging from 0.1 to 1000 ng/mL labeled standard.

Each solution was analyzed by LC-MRM/MS in triplicate, and the data analysis allowed the selection of AMH (209−219) peptide as *quantifier* for the quantitative analysis of AMH. This peptide showed the best instrumental response over a wider linear concentration range (0.1−500 ng/mL).

The calibration curve obtained by plotting the peak area as a function of heavy standard peptide concentration (ng/mL) for the GSGLALTLQPR* peptide is reposted in Figure S1A, as well as the visualization of the coelution of TIC recorded for the heavy peptide and the endogenous light one at 8.1 min (Figure S1B).

All the analytical parameters, LOD, LOQ, working range, upper limit of the working ranges, calculated y-intercept, and angular coefficient obtained for the calibration curves were determined and are reported in Table 1.

Table 1. Analytical Parameters to Validate the Quantification Method for AMH Using LC-MRM/MS and Heavy Standard Peptides

parameter	value
m	67799.25
q	−20783
R^2	0.9945
working range	0.1−561 ng/mL
upper limit of working range	561 ng/mL
LOD	0.08 ng/mL
LOQ	0.24 ng/mL
%Accuracy	96.5−104.7%
%Recovery	22%
%RSD	10.5%

The matrix effect was evaluated by spiking a pool of serum samples (10 μL) with a known amount of the heavy standards (250 ng/mL). The spiked pool of sera sample was treated as reported in section 4 and then analyzed 10 times on the same day and in the same conditions to evaluate the effect of interfering substances on AMH quantification to evaluate the precision and the accuracy.

The determination of a low recovery value reflects the high complexity of serum as a biological matrix.

Data normalization was performed toward protein quantification using the Bradford assay, which allowed the determination of an average protein concentration of 73.42 ± 3.65 μg/μL according to literature data[19] with a good calibration curve (R^2 that resulted to be 0.998), suggesting a strong correlation between concentration and instrumental response.

The 54 serum sample cohort was then analyzed to test the LC-MS/MS method in real samples. The samples were subjected to trypsin hydrolysis by using the S-Trap protocol, the peptide mixture was analyzed in triplicate, and the AMH quantification results were determined as averages of the three replicates (Supplementary Table 2). The technical variation among replicates, expressed as the coefficient of variation (CV %), suggested a good precision of the method toward recording values lower than 15% for all the analyzed samples. The units of measurement for the determination of AMH were expressed in ng/mL to standardize the mass spectrometry data with those of the ELISA assays currently used in clinical laboratory.[15]
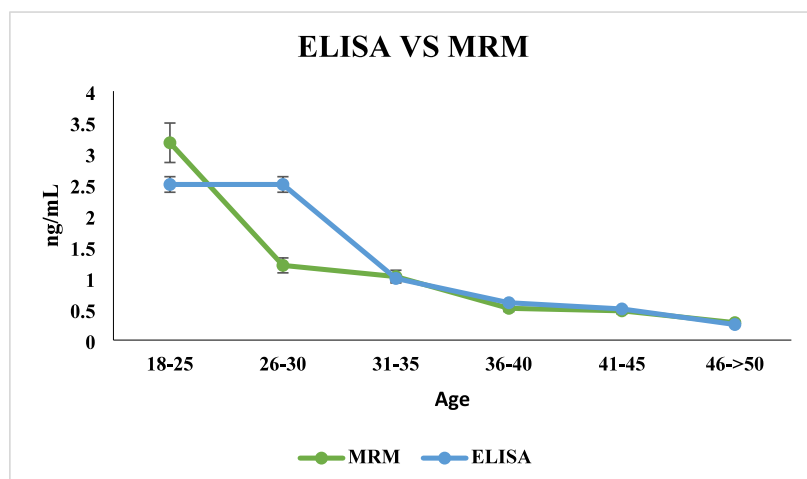
Figure 1. Trend of AMH levels in serum samples pooled in agreement with age by using LC-MRM/MS (green line) and ELISA (blue line).
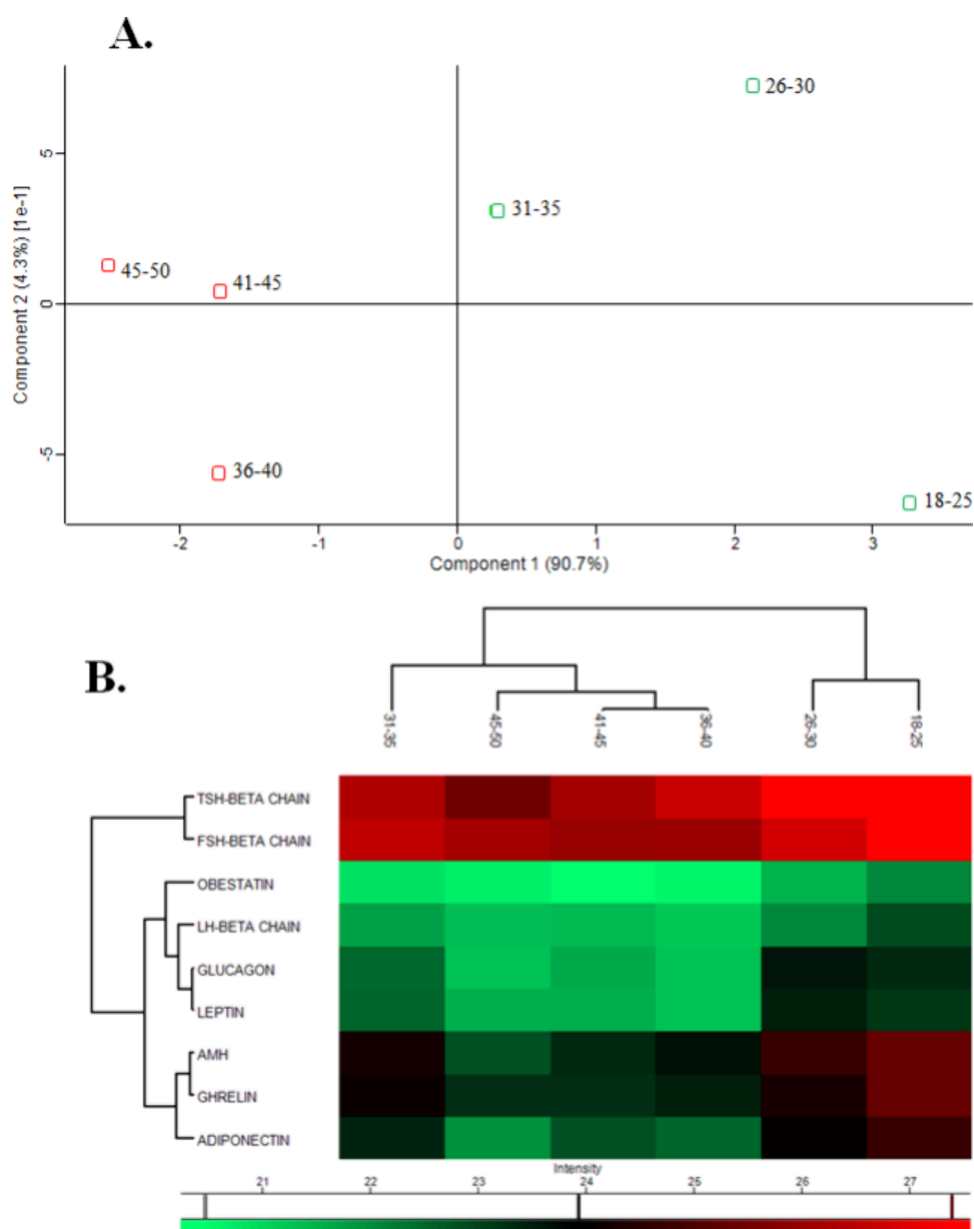

Figure 2. Perseus statistical evaluation of LC-MS/MS data obtained by PCA analysis (A) and heat map visualization (B).

The comparison between the data obtained using the two techniques is shown in Figure 1 where the AMH concentration expressed as ng/mL wasis reported as a function of the age of the women. The trend obtained through LC-MS/MS analysis was similar to the ELISA assay, except for the AMH concentration in the age ranges of 18−25 and 26−30. Most likely, an increase in the number of samples in the 26−30 age group could shorten the distance between the two methods due to biological variability. For almost all samples, it was possible to quantify the amount of AMH, and the values obtained faell within the ranges also determined with other ELISA assaysys, confirming the potential of the MRM approach.[20]

The trend obtained for the AMH concentration reflected what others have reported regarding the decline in AMH values with age. The literature is controversial regarding the use of AMH as a marker of a woman's fertility. It is noteworthy that ovarian aging is detected as a decrease in the quality and quantity of the ovarian follicular pool, especially with aging.[21] Furthermore, it is highlighted how ovarian aging is related to a decrease in the ability to conceive spontaneously and the limited success of IVF treatments with advancing age.[22,23] Poor ovarian reserve is commonly reported in women in their thirties but can also affect younger women.[24] The reduction in the number of ovarian follicles has been described as a slow decline from birth to age 38 followed by a rapid decay.[14,25]

Another important aspect of recent interest revealed the link between AMH decrease and ethnic factors. Indeed, AMH decrease is more significant in Chinese women than in Caucasian ones, reflecting decreases in the AMH value of 28% and 80% at ages of 35 and 40, respectively, as reported by Nelson et al.[26] These variations could be associated with genetics, nutrition, and environmental influences.[27] Another limiting factor in the use of AMH as an ovarian marker is the apparent inability to measure age-specific levels in various ethnic groups. One of the advantages of measuring the serum AMH level is the minimal variation between and within menstrual cycles; therefore, it can be measured on any day of the menstrual cycle.[28] Other authors have already proposed a method for the analysis of this protein, managing to quantify it by using three different isotopically labeled peptides but only in standard solutions prepared by spiking with a known amount of AMH.[29] The present Article was proposed as a next step in the development and application of the method to serum samples from women of different ages. In addition, a comparison between AMH levels determined by MRM/MS and those resulting from enzyme immunoassays for the same subjects was conducted by demonstrating a good correlation between the results obtained with the two methodologies.

2.2. Relative Quantification of Gonadotropins and Adipokines.

Once the method for determining serum AMH levels was finalized, other proteins were also added to the method to exploit the wide versatility of MRM tandem mass spectrometry: FSH and LH belonging to the gonadotropin class; TSH and glucagon as hormones produced by thyroid and pancreas, respectively; and adiponectin, ghrelin, obestatin and leptin classified as adipokines. The simultaneous quantification of AMH and gonadotropins together with adipokines also involved in fertility is crucial to get a clearer understanding of the clinical state of a woman's health.

The method for the determination of adipokines is currently under review, and only a relative quantification was performed among all samples. Then, the protein abundances expressed as areas of MRM chromatogram peaks were compared by multivariate analysis performed on Perseus software. The statistical analysis produced a data visualization based on PCA and the hierarchical clustering (heat map) (Figure 2). As evinced by Figure 2, the first two principal components were used to obtain the PCA biplot capable of reducing the changes in abundance among samples by explaining 95% of variance. Only 5% of variance remained to be included in the other three components. Good clustering of data points was visualized in agreement with the age range, allowing two major regions of PCA biplot to be distinguished defining the sera of women under 35 years old and over 35 years old. Even the heatmap enabled the visualization of the same clustering obtained by PCA toward the representation of changes in protein abundance along a color scale of different intensity.

The graphical visualization of the results showed a clear clustering between the data obtained from the pools of women under and over 30 years of age. The heatmap allowed the visualization of a general decrease in all monitored hormone levels with aging. Furthermore, the highest peak areas were recorded for TSH and FSH. These results supported previous finding by Mancuso et al. in which aging alters both the composition and function of adipose tissue, leading to a decrease in the levels of sexual hormones and an increase in abdominal adipose tissue due to the redistribution of lipids from the subcutaneous compartment to the visceral one.[30] These changes are correlated with a chronic state of low-grade systemic inflammation.[31,32] Although the decrease in the level of gonadotropins was expected with advancing age, alterations in adipokine levels were recorded at the same extension. The changes in abundance as well as the altered mechanism of action were reported to be associated with fertility disorders and pregnancy diseases beyond obesity, metabolic syndrome, and cardiovascular diseases. Normal levels of adipokines are critical for maintaining the integrity of the hypothalamic−pituitary−gonadal axis, for regulating ovulatory processes, ovarian steroidogenesis, oocyte maturation, and embryo development, for the success of embryo implantation and physiological pregnancy.[33−36] Adipokines have also been identified in the uterus and placenta.[37,38] Some studies also suggested that the follicular leptin/ghrelin ratio is a suitable indicator for predicting IVF outcomes in women with normal body mass indexes.[39]

3. CONCLUSIONS

ELISA assays are the elective techniques in clinical investigation for determining the dosage of protein hormones, mainly for their high diagnostic sensitivity, specificity, and versatility in analyzing any kind of biological matrix. Such an approach may suffer from the reduced reliability due to the antibodies' cross-reaction or to the detection of interferants, giving rise to erroneous results. Due to the urgency of pushing the analytical practices toward molecularly more specific techniques with greater speed of analysis to quantify numerous molecules in biological samples, researchers and clinicians alike are now turning to mass spectrometry as an alternative analytical technique.

Recently, LC-MS/MS analysis in the MRM ion mode has proven to be a valid alternative to currently used techniques thanks to the limit of detection and quantification comparable to those of ELISA assays. The advantage of using an LC-MRM/MS method comes from the potential to simultaneously monitor eight protein hormones involved in fertility, including

AMH, in a single run with high reproducibility and selectivity beyond reduced cost and time. LC-MS/MS in the MRM mode offers an inherent specificity advantage, primarily the molecular analysis based on peptide fragmentation, as well as multiplexing capabilities, albeit with significant start-up costs.[40] Indeed, despite the initial expense of purchasing the instrument, the costs of LC-MRM/MS-based analyses are lower than those for the ELISA assay.

This aspect opens the way to the use of mass spectrometry in routine analysis to support the diagnosis and treatment of ovarian and infertility conditions.

4. MATERIALS AND METHODS

4.1. Materials. The following reagents from Merck were used for the analysis: BSA (bovine serum albumin), dithiothreitol (DTT), iodoacetamide (IAM), methanol (MeOH), AMBIC (ammonium bicarbonate), leptin, glucagon, obestatin, ghrelin, adiponectin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and sodium dodecyl sulfate (SDS). Acetonitrile (ACN) and phosphoric acid were from Fluka; formic acid (HCOOH) was 99% from Carlo Erba. The S-Trap Mini Spin was from Protifi, and the Bradford reagent assay was from BioRad. Synthetic isotopically labeled peptides were purchased from Thermo Fisher.

Two cohorts of serum samples were used to conduct this study: 54 serum samples provided by the Experimental and Clinical Senologic Oncology Unit, Istituto Nazionale Tumori, IRCCS Fondazione G. Pascale, Napoli, to improve the sample treatment and method development. Moreover, another cohorts of 79 serum samples was provided by Istituto Varelli for method validation thanks to the availability of AMH values determined by ELISA. All the samples involved in this project are from women aged between 20 and 60 years, and the processing of the samples was accompanied by informed consent of the patients.

For method development, all the samples from the first cohort were used, while 9 pools were created according to the women's age as reported in Table 2 for method validation.

Table 2. Pool of Serum Samples Realized According to Women's Ages for LC-MRM/MS Analyses

age	samples
18−25	9
26−30	10
31−35 (1)	10
31−35 (2)	10
36−40 (1)	7
36−40 (2)	7
41−45 (1)	7
41−45 (2)	7
45−50	12

4.2. Methods. *4.2.1. Protein Digestion on S-Trap Columns.* After the protein quantification performed by the Bradford assay,[41] each sample was subjected to protein digestion on an S-Trap column as previously described.[42] Briefly, serum (10 μL) was added to an equal volume of lysis buffer (10% SDS and 100 mM AMBIC). Then, 100 mM DTT (5 μL) was added to the protein sample to reduce disulfide bridges, and the reaction was carried out in a 95 °C thermostatic bath for 10 min. After the samples were cooled,

40 mM IAM (8 μL) was added to alkylate the thiol residues generated during reduction, and samples were incubated in the dark for 30 min at room temperature. A solution of 12% phosphoric acid was added to the mixture in a 1:10 ratio to halt the alkylation process. Then, the samples were centrifuged at 12,000 rpm for 8 min to recover the supernatant. The protein mixture was then diluted with 180 μL of S-Trap buffer (100 mM AMBIC in 90% MeOH and 10% H_2O) and loaded on the S-Trap column. To adsorb the proteins onto the column bed, the solutions were centrifuged at 5000 rpm for 3 min. Three washes were performed with 250 μL of S-Trap buffer, centrifuging at 5000 rpm for 3 min after each addition. For enzymatic digestion, 125 μL of 0.12 μg/μL trypsin dissolved in 10 mM AMBIC was added to each sample, and rapid centrifugation was performed to allow trypsin to penetrate the S-Trap. The gathering tubes were replaced, and the reaction was conducted for 2 h at 47 °C in a thermostatic bath. Several washes were performed with different solutions to elute and collect all the peptides generated by trypsin digestion through centrifugation at 5000 rpm for 3 min: the first consisted of 80 μL of 0.2% HCOOH; the second elution was performed with 80 μL of a solution of 50% ACN, 50% H_2O, and 0.5% HCOOH; and the final elution was performed with 80 μL of a solution of 80% ACN, 20% H_2O, and 0.5% HCOOH. Each sample was then subjected to a further desalting procedure using stage tips containing three layers of 3 M Empore C18 membrane (Supelco). Stage tips were washed with 100 μL of 0.1% HCOOH, and peptides were eluted with 50 μL of 50% ACN and, subsequently, with 80% ACN, both acidified with 02% HCOOH. The eluate was dried using a speed vac concentrator and stored for further mass spectrometry analysis.

4.2.2. Internal Standard Method. For the matrix calibration curve, seven aliquots of 10 μL of women's serum sample (18−25 age) were used for the S-Trap digestion. The samples were then spiked with standard AMH heavy (209−219) peptide of sequence GSGLALTLQPR*(*$^{13}C_6$; $^{15}N_4$) to have different peptide concentrations in the seven different pools (1000−5 ng/mL). A solution of 2% ACN containing 0.2% HCOOH was added to spiked samples to reach a final volume of 50 μL, and the stage tip protocol was performed.

Then, each serum pool was prepared by spiking a known amount of standard heavy peptide (250 ng/mL) to calculate the recovery in the matrix.

4.3. LC-MS/MS Analysis. The samples were resuspended in 30 μL of a solution of 2% ACN containing 0.2% HCOOH and analyzed by LC/MS-MS. Peptide mixtures were analyzed by LC-MS/MS analysis using a Xevo TQ-S (Waters) instrument equipped with an ionkey coupled to an Acquity UPLC system (Waters, Milford, MA, USA). For each run, 2 μL of peptide sample was injected and separated on a BEH C18 peptide separation device (130 Å, 1.7 μm, 150 μm × 50 mm) at 45 °C with a flow rate of 3 μL/min using an aqueous solution (LC-MS grade) containing 2% ACN as buffer A and 98% ACN as buffer B, both acidified with 0.2% HCOOH. The gradient for the MRM method started with 7% buffer B for 5 min, reached 50% buffer B from 5 to 40 min, and reached 95% buffer B during the next 2 min. The column was finally re-equilibrated to initial conditions for 4 min. The parameters of the MS source were as follows: 3900 V as the ion spray voltage, 150 °C interface heater temperature, and 150 L/h gas flow with 7 bar nebulizer pressure.

MRM mass spectrometric analyses were performed in the positive ion mode for the run time with 5 points per peak and dwell times of 3 ms. The cone voltage was set to 35 V. A range of 300−1000 m/z was preferentially selected to choose the precursor or product ions (m/z) for each target peptide.

All instrumental parameters, e.g., the selected peptides for each protein target, precursor and product ions (m/z), collision energy (eV), and cone voltage (V), are included in Table S1. To set up the LC-MS/MS method, softwares like SRMAtlas and Skyline[43] (version 23.1.0.455) were consulted by using the Uniprot id to select the best transitions for extracting the MS parameters and for the data visualization.

LC-MS/MS output files were processed by using thePerseus (version 1.6.8.0)[44] software platforms to create a graphical representation of the obtained mass spectrometry data. By using this software, the intensity values expressed as peak areas related to each protein group were log-transformed to $\log_2(x)$. PCA and the heatmap were realized by using the Perseus matrix of data.

4.4. Method Validation. To quantify AMH using the internal standard method, calibration curves were built up by spiking seven pools of serum samples (10 µL) with increasing amounts of the heavy standard peptide.

Each point of the calibration curve in the range of concentration of 1000−5 ng/mL was analyzed in triplicate by using the LC-MRM/MS method. The upper limit of the working range was defined for each selected standard compound as the concentration at which the instrumental response became nonlinear. The limit of detection (LOD) and limit of quantification (LOQ) were estimated using the following equations: $\text{LOD} = 3.3 \times \frac{\sigma}{b}$ and $\text{LOD} = 10 \times \frac{\sigma}{b}$, where σ is the standard deviation of the y-intercept and b is the angular coefficient of the calibration curves calculated for each standard peptide. To evaluate repeatability and accuracy, a 250 ng/mL mixture of standards was analyzed 10 times ($n = 10$) on the same day and under the same conditions. Therefore, repeatability was calculated as relative standard deviation (% RSD) according to

$$\%\text{RSD} = \frac{\sigma}{c_{\text{average}}} \times 100$$

where σ is defined as the standard deviation of the 10-replicate analysis and c_{average} represents the average AMH concentration calculated for the 10-replicate analyses. Accuracy was defined as $\%\text{Accuracy} = \frac{c_{\text{exp}}}{c_{\text{std}}} \times 100$, where c_{exp} is the quantification results and c_{std} is the known concentration of the standard analyte. The matrix effect of the quantification of AMH was evaluated as the recovery value of standards as reported in the equation

$$\text{Recovery} = \frac{C_{\text{spike}}}{C_{\text{std}}} \times 100$$

where c_{spike} is the analyte concentration measured in the spiked serum sample pool and c_{std} is the analyte concentration measured in a non-spiked pool of serum.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02250.

MRM/MS method for monitoring the selected targeted proteins, AMH level in serum samples determined by LC-MRM/MS, calibration curve for AMH 209−219 GSGLALTLQPR (PDF)

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Notes

The authors declare no competing financial interest.

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