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Immunoassay

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1. Introduction

Anti-Möllerian hormone (AMH) is a glycoprotein that belongs to the transforming growth factor-β (TGF-β) superfamily [1]. AMH, also known as a Möllerian inhibiting substance (MIS), has been mainly studied for its regulatory role in male sex differentiation and induces regression of the Möllerian ducts, the anlagen of the female reproductive tract [2]. AMH expression can first be observed in granulosa cells of primary follicles, and its expression is strongest in preantral and small antral follicles (<4mm). AMH expression disappears in follicles as their size increases and is almost completely lost in follicles larger than 8mm, where only very weak expression remains, restricted to the granulosa cells of the cumulus [3]. This expression pattern suggests that, also in women, AMH may play a role in the initial recruitment and in the selection of the dominant follicles. To assess an individual's ovarian reserve, early follicular phase serum levels of FSH, inhibin B and estoradiol (E_2) have been measured. Inhibin B and E_2 are produced rarely by antral follicles in response to FSH, and contribute to the classical feedback loop of the pituitary-gondola axis to suppress FSH secretion. With the decline of the follicle pool, serum levels of inhibin B and E_2 decrease and subsequently serum FSH levels rise [4].The likely explanations are that AMH and FSH are highly correlated [5]. In women undergoing treatment for infertility, ovarian aging is characterized by decreased ovarian responsiveness to exogenous gonadotrophin and poor pregnancy outcome. On the one hand, correct identification of poor responders by assessment of the ovarian reserve before entering an IVF program is important. On the other hand, assessment of the ovarian reserve may also benefit patients that would generally be excluded from an IVF program because of advanced age.

Automatic follicle analysis has the potential to remove any observer bias and to reduce the time needed for measurements, but it must be both valid and reliable [6]. This is particularly true within the field of reproductive medicine, with ultrasound being used on a daily basis to follow the ovarian response to gonadotropins in a process known as follicle tracking [7]. Two-dimensional (2D) measurements are then made and their mean is taken as the true follicular diameter, which is relatively straightforward but follicle tracking becomes more difficult as the number of follicles increases [6]. Therefore, recently, three-dimensional (3D) ultrasound with Sono AVC (Automatic Volume Calculation) has been used to quantify hypoechoic regions within a three-dimensional dataset and provide automatic estimation of their absolute dimensions, mean diameter and volume. Each individual volume is given a specific color and the automated measurements of the mean diameter (relaxed sphere diameter), maximum dimensions (x, y, z diameters) and volume are displayed using these

colors in descending order of size. Sono AVC provides measurements of follicular diameter that are more accurate than manual measures and has the potential to improve the clinical workflow because the time taken for the measurements is significantly shorter [8]. The quantification of power Doppler assesses blood flow within the ovary and quantitative 3D-power Doppler angiography has been used to demonstrate the blood flow around the follicles within the ovary. These tools are new methods to analyze the effect of ovary stimulation. The power Doppler data within the 3D dataset can be quantified to generate volumetric measures of blood flow within the dataset as whole or within specific volumes within the dataset. Various software programmes are available to facilitate this, but the one used most frequently is the histogram facility in 4D View (GE Medical System, Zipf, Austria), which generates three indices of vascularity: the vascularization index (VI), the flow index (FI), and the vascularization flow index (VFI). These vascular indices are generated through specific algorithms based on signal intensity and the relative proportion of color voxels (3D pixels) within the defined volume [9]. This study was designed to assess the ability of the combination of AMH and 3D-power Doppler histogram techniques, including the Virtual Organ Computer-aided (VOCAL) software, to generate volume measurements of the ovary. These new combinational ultrasound techniques were used to investigate patient undergoing IVF treatment.

2. Materials and methods

This was a prospective cohort study of 28 patients undergoing controlled ovarian hyperstimulation**.** All of the patients met the following inclusion criteria: (1) Both ovaries were present with no morphological abnormalities, (2) Regular menstrual cycle lengths ranging between 25 and 34 days, (3) No current or past diseases affecting the ovaries or gonadotrophin or sex steroid section, clearance, or excretion, (4) No clinical signs of hyperandrogenism, (5) Body Mass Indexes ranging from 18 to 25kg/m^2, (6) No current hormone therapy, (7) Adequate visualization of both ovaries in transvaginal ultrasound scans. Informed consent was obtained from all patients and this investigation was approved by our internal Institutional Review Board.

2.1 IVF treatment

Patients were treated with a time-release GnRH agonist from day 2 of menses (Day2). On Day 3, complete pituitary desensitization was confirmed by the detection of low serum levels of E_2 and gonadotrophins. Patients also underwent a conventional ultrasound examination to exclude ovarian cysts and to verify that the endometrium was <5mm. Recombinant FSH therapy (recombinant Follitropin beta) or HMG was then initiated at a dose of 150 IU/day, while daily Gn-RH agonist administration was continued until the day of administration of hCG at 10,000 IU dose.

2.2 AMH

Blood was sampled between days 1 to 16 of menses (Day 1 to 16) from women undergoing IVF treatment. Follicular fluid was obtained from the follicles at Day 14. Blood and follicle fluid were centrifuged at 3,000 bpm/min for 10 minutes, and then stored at -20°C until immunoassay. Serum AMH (S-AMH) levels were assessed using enzyme immunoassay AMH/MIS-EIA (IMMUNOTECH A BECKMAN COULTER COMPANY). Recombinant

human AMH was used as a calibration standard to generate a standard curve (conversion factor to pmol/l=ng/ml×7.14).

2.3 3D ultrasound methods.

Ultrasound image sampling was performed on 28 patients undergoing IVF treatment from Day 1 to 16. All assessments were performed using a ultrasound machine and a four-dimensional 9MHz trans-vaginal probe. This provides visualization of the three orthogonal planes so that the central point of the follicle within the right ovary (RO) and the left ovary (LO) were consistent for all three images.

2.4 Sono AVC

The Sono AVC was activated when it had been correctly positioned and magnified. The setting of growth and separation within the software was maintained at a default value of mid for all follicle measurements. The Sono AVC identifies the follicle by giving it a specific color (Figure 1a) and provides automated measurements of the mean diameter (relaxed sphere diameter), maximum dimensions (x y z diameters) and volume (Figure 1b).

2.5 Virtual Organ Computer-aided (VOCAL) and 3D power Doppler volume histogram

3D power Doppler volume histogram was generated based on the VOCAL method. The stimulated ovary was used to measure each index of blood flow in vasculature and vessels per gray scale ratio in the ovary, resulting in the Vacularization Index (VI), Flow Index (FI) and Vascularization Flow Index (VFI). These indexes display on the volume histogram (Figure 2).

2.6 Statistical analysis

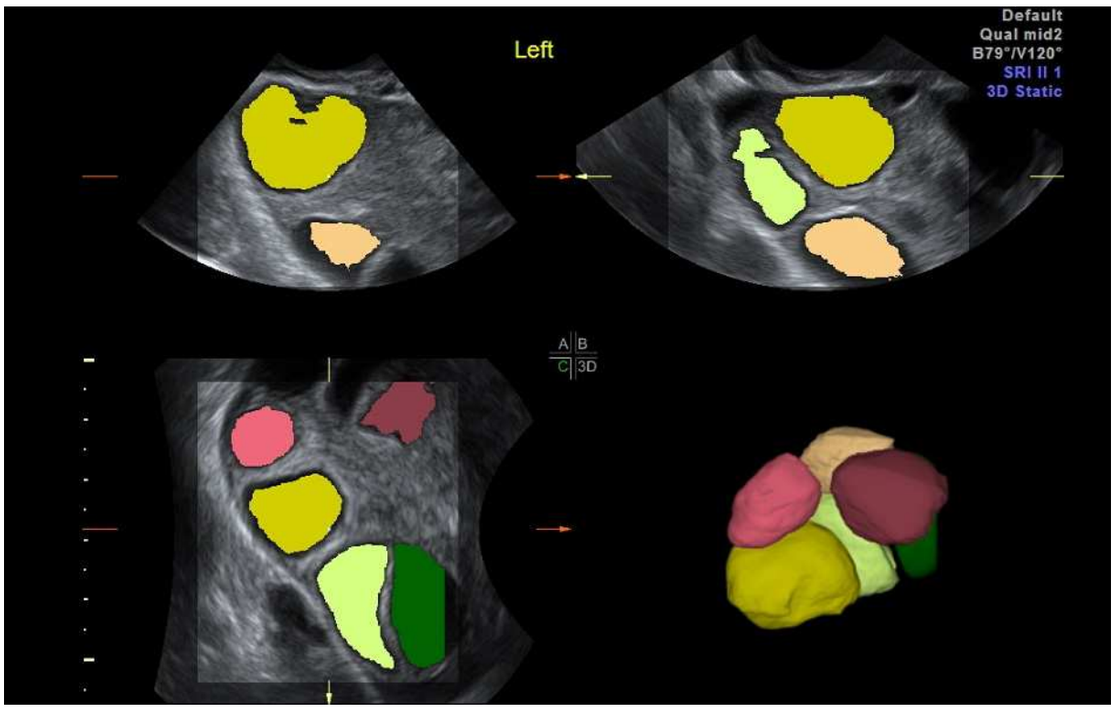
Student's *t*-test and Fisher's exact test were conducted using Stat View 5.0 (Abacus Concepts, Inc. Berkeley CA, 1996). Principal component analysis (PCA) and Receiver operating characteristic (ROC) curves were calculated using SPSS 17.0 (SPSS Japan Inc).

3. Results

This study population consisted of 28 patients. The age range was 25 to 44 years (under 35 years: n=6, 35-39 years: n=8, over 40 years: n=14), and the mean age was 36.74±5.88 years. Blood samples were obtained from each patient and 3D ultrasound examination was performed at Day 10.04±5.8 (range: 1 to16). The number of oocytes aspirated was 5.6±3.6 (range; 0 to 16), and the number of embryos generated was 3.3±2.8 (range: 0 to 11).

3.1 AMH

The mean S-AMH level was 0.47±0.125 ng/ml, and the level in the patient under 35 years of age undergoing IVF treatment (0.570±0.216 ng/ml) was significantly higher than that in patient over 40 years of age (0.377±.070 ng/ml; p=0.0003) (Figure 3a), and the mean S-AMH level which was 0.469± 0.181 ng/ml in 35 to 39 years of age. The relationship between the mean S-AMH levels and age of women undergoing IVF treatment was significant (r=0.459, p=0.0004. 95%CI: -0.23 to 0.07) (Figure 3b). These results show that S-AMH levels reduced with the increasing age of women undergoing IVF.



(a)

LMP	2008/10/01	Day of Cycle	12	Gravida		AB	
Day of stim.		Expected Ovul.		Para		Ectopic	

Ovary:	Left						Ovary:	Right					
Total#:	9						Total#:	9					
Nr.	d(V) mm	dx mm	dy mm	dz mm	mn. d mm	V cm³	Nr.	d(V) mm	dx mm	dy mm	dz mm	mn. d mm	V cm³
1	18.9	25.2	20.2	14.6	20.0	3.55	1	18.9	25.2	20.2	14.6	20.0	3.55
2	18.0	20.3	18.2	16.8	18.4	3.07	2	18.0	20.3	18.2	16.8	18.4	3.07
3	17.5	30.4	15.9	13.6	20.0	2.82	3	17.5	30.4	15.9	13.6	20.0	2.82
4	16.0	26.4	16.1	12.1	18.2	2.16	4	16.0	26.4	16.1	12.1	18.2	2.16
5	15.8	20.9	16.3	12.1	16.4	2.07	5	15.8	20.9	16.4	12.1	16.4	2.07
6	15.8	22.3	19.5	10.1	17.3	2.07	6	15.8	22.3	19.5	10.1	17.3	2.07
7	14.7	18.4	15.9	11.6	15.3	1.66	7	14.7	18.4	15.9	11.6	15.3	1.66
8	12.9	19.0	15.1	9.5	14.5	1.14	8	12.9	19.0	15.1	9.5	14.5	1.14
9	8.7	13.0	8.3	6.8	9.4	0.35	9	8.7	13.0	8.3	6.8	9.4	0.35

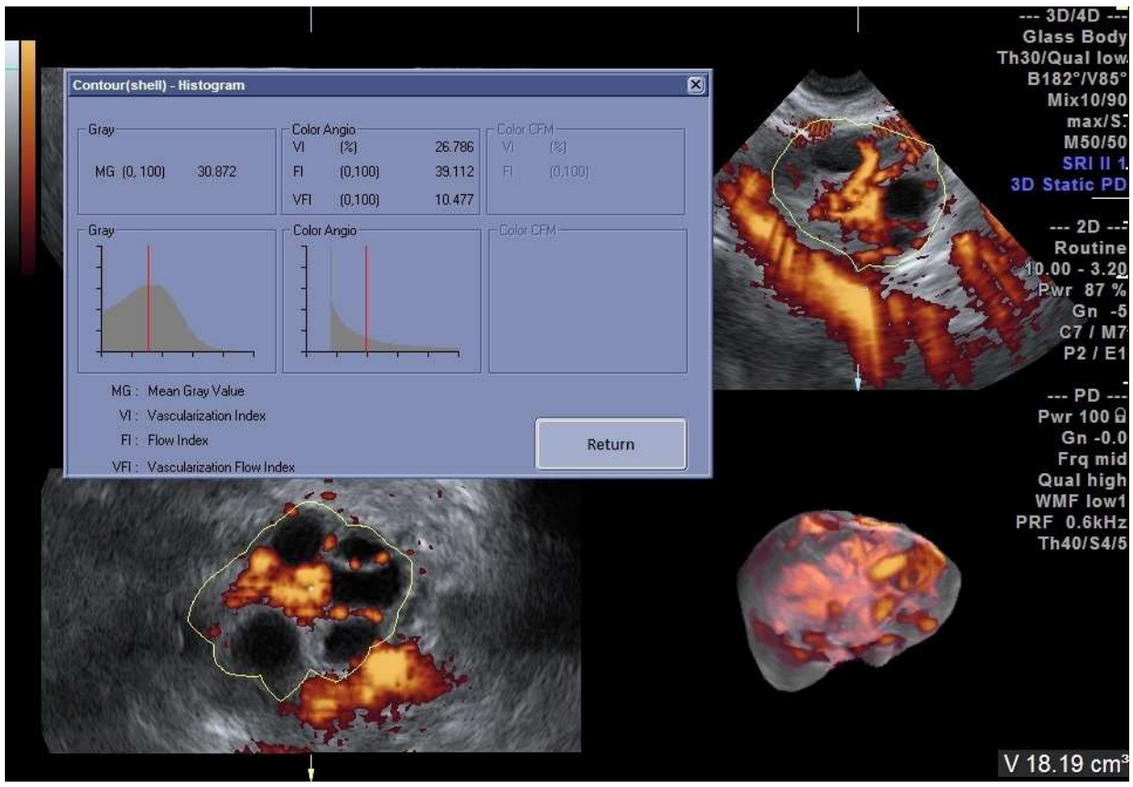
Pelvic Floor

funneling	yes	no
urethral kinking	yes	no

(b)

1a The Sono AVC identifies the follicle by giving it a specific color
1b many follicles automatically were measured that displayed report on monitor

Fig. 1. Automatic volume calculation (Sono AVC) was used to automatically calculate the volume of the follicle.



Three-dimensional power Doppler histogram was used to determine the vascular and blood flow, Vascularization indices (VI; vascularization index, FI; flow idex, VFI; vascularization flow index) from computer algorithms.

Fig. 2. Three-dimensional power Doppler image around follicle in ovary.

3.2 3D ultrasound methods

3.2.1 Sono AVC

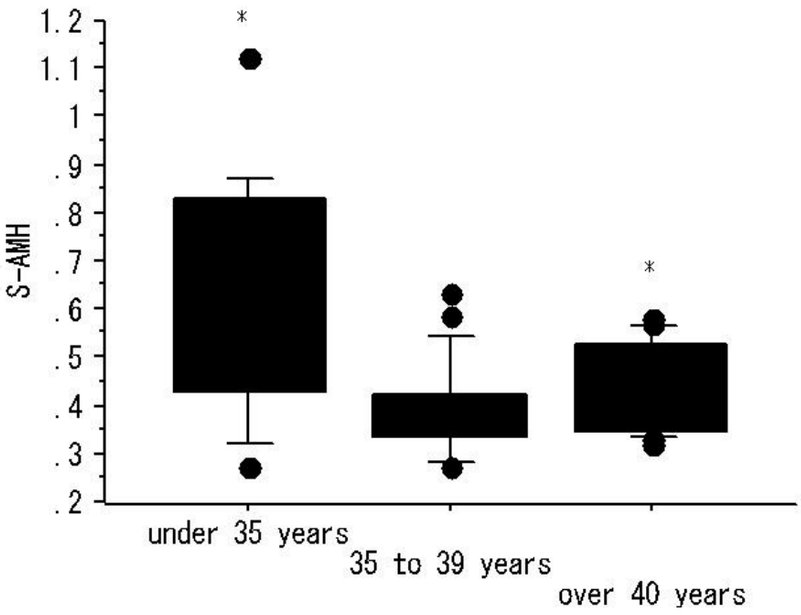
The mean number of follicles was 5.61±3.28 from the right ovary (range: 0 to 13) vs. 5.46±4.56 from the left ovary (range: 0 to 19). The mean volume of the right follicle was 0.6999±0.613 cm^3 (range: 0.0 to 2.846; cm^3), and the mean volume of left follicle was 0.675±0.845 cm^3 (range: 0.0 to 3.220; cm^3).

3.2.2 3D-power Doppler Volume histogram

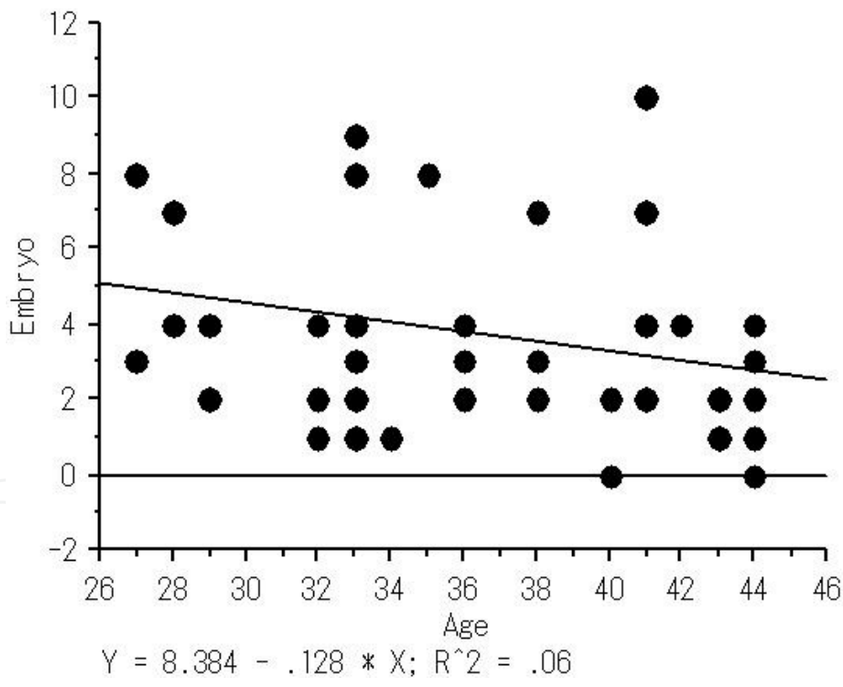
The mean FI of the RO and LO were 34.39±9.897 and 29.88±19.66%, respectively. The mean VI of the RO was significant higher LO (RO vs., LO; 7.61±1.121 vs., 3.30±0.679, p=0.013), and the mean VFI of the RO was high compare with LO (RO vs., LO; 2.37±0.337 vs., 0.776±0.844, p=0.024). The mean FI was no difference of index between RO and LO. 0.924) and decreased the S-AMH (oocyte 0.583, embryo 0.647).

3.2.3 The ROC curve

In the embryo, the cut-off value for the S-AMH was 0.2855 ng/ml (AUC; 0.56, sensitivity, 83.3%. specificity, 92.9%; 95% CI, 0.268 to 0.851) (Figure 4a) and the optimum cut-off point



(a)

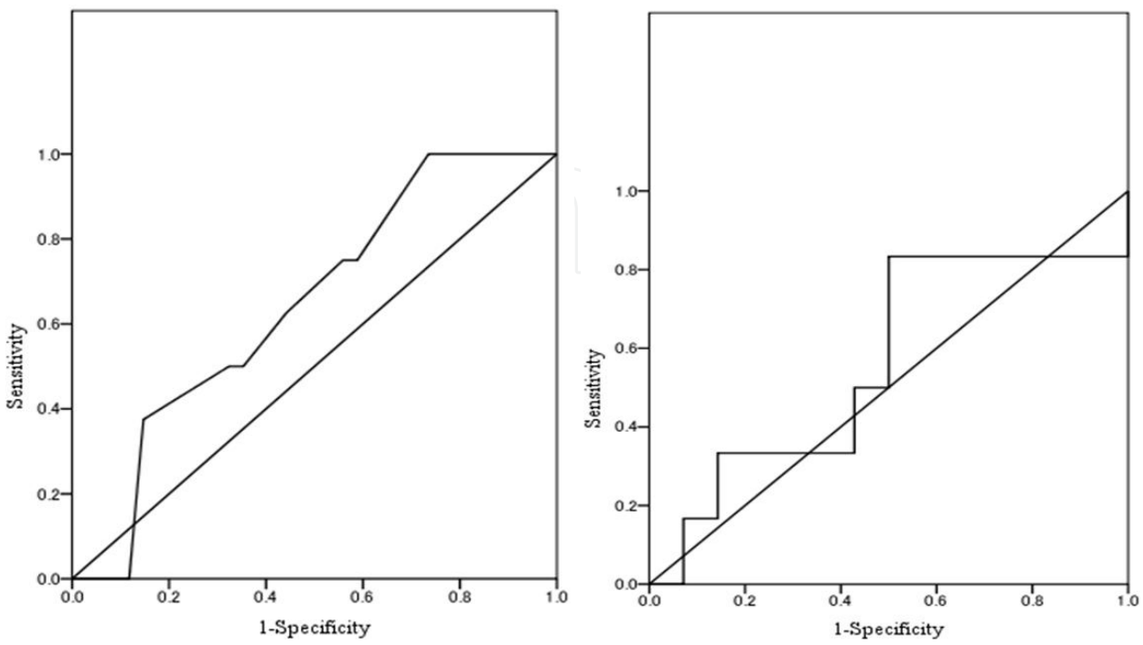


(b)

3a Under 35 years of age of patient (0.47±0.125) was significantly higher than over 40 years of patient (0.377±.070) (p=00003). 35 to39 years of age was 0.570±0.216 ng/ml.
*p<0.05compared to three different group by Fisher's exact test
3b Serum-AMH level corrected for age of patient undergoing *in vitro* fertilization treatment
r=0.459, p=0.0004.295%CI: -0.23 to 0.07 by linear regression.

Fig. 3. Serum-AMH level (ng/ml) relation with age of patient undergoing *in vitro* fertilization treatment

for the number of embryo was an age of 32.5 years (AUC; Sensitivity; 100%, Specificity; 52.6%, 95% CI 0.542 to 0.892) (Figure 4b).



(a) (b)

4a ROC curve that Serum-AMH of cut -off value which took embryo relation with lime of age was 0.2855ng/ml (AUC 0.56. sensitivity, 83.3%. specificity, 92.6 %; 95% CI, 0.268-0.851) (Figure 5c).
4b ROC curve that embryos were taken for the lime of age was 32.5 years (AUC; 0.62, Sensitivity; 100%, Specificity; 52.6%, 95% CI 0.542 to 0.892) (Figure 5d).

Fig. 4. The Receiver-operating characteristic (ROC) curve indicate cut-off value.

4. Discussion

4.1 AMH

In this study, we investigated whether AMH and measurements could be useful markers of successful generation of embryos from patients undergoing IVF treatment, and examined the relationship with the embryo using 3D ultrasonography. We further tested the diagnostic performance of AMH and the age limit of patients undergoing IVF treatment on the number of embryos obtained, by ROC curves. These data led to the conclusion that AMH, Sono AVC and 3D-power Doppler histogram could provide the best available clinical markers of responsiveness to the ovarian stimulation. Our results indicate that AMH is suitable as an effectiveness factor, such as the generation of embryos with IVF treatment, by principal component analysis. We also found that the S-AMH cut-off value was 0.2855 ng/ml (AUC: 0.56. sensitivity, 83.3%. specificity, 92.9%; 95% CI, 0.268 to 0.851) in all cases and the optimum age cut-off was 32.5 years (AUC: 0.643. sensitivity, 100%. Specificity, 52.6%; 95% CI, 0.453 to 0.833). In 2007, Zappacosta et al. reported the Bland-Altman plot analysis (AxSYM Abbott) shows that the immunonephelometric method has a slight positive bias with both HPLC (mean: 1.03µmol/L, 95% confidence interval:0.28-1.79µmol/L)

and AxSYM methods (mean: 0.45μmol/L, 95% confidence interval:-0.03-0.94μmol/L) AMH is a significantly correlated with ovarian response in IVF cycles. Indeed, linear regression analysis shows a significant association between AMH and the number of ovocytes collected [11]. AMH levels in women with correct ovarian response range from 0.63 ng/ml [12] to 0.67ng/ml [13]. Scott et al. demonstrated that a single measurement of circulating AMH can be used to individualize treatment strategies for IVF, potentially resulting in reduced clinical risk, along with optimized treatment burden, and clinical pregnancy rates, with the application of the Gn-RH antagonist protocol appearing to be advantageous for patients at the anticipated extremes of ovarian response [14]. In 2009, Singer et al. reported that the use of FSH and AMH in combination might improve the evaluation of ovarian reserve. However, it remains to be determined which of these two ovarian function parameters is superior in assessing ovarian reserve with a single test and which test, or combination of tests is most appropriate [5]. In our study, we did not demonstrate to determine FSH and AMH in combination, and the relationship between S-AMH and successful pregnancy, we think that the reasons which are the first, it is most important determine AMH level compare with FSH level, because FSH levels are effected of FSH stimulate ovarian function undergoing IVF treatment, and the second, likely pregnancy has many factors, including hormones, the immune-cytokine network and the developing syncytium of cells in the endometrium of the uterus. Therefore, the aim of our study was clearly distinct from these studies, as we assessed the combination of AMH and 3D ultrasound methods that automatically measure follicles and blood flow in ovaries during IVF treatment. Particularly, we indicated that the S-AMH relation with the age during IVF treatment from the limit of the S-AMH was 0.2855ng/ml and the number of embryos (≥2) with an optimum cut-off age of 32.5 years. We believe that these cut-off values are predictive of a poor response undergoing IVF treatment. Finally, it is nasally to analyze S-AMH levels and to consider age undergoing IVF treatment. Gnoth et al reported that cut-off that AMH is a predictor of ovarian response and is suitable for screening. A calculated cut-off level≤1.26ng/ml AMH alone detected poor responders (≤4 oocytes) with a sensitivity of 97%, and there was a 98% correct prediction of normal response to IVF treatment if levels were above this threshold. With levels of 0.5ng/ml, a correct prediction of very poor response (≤2 oocytes) was possible in 88% of cases. The Gnoth group report that AMH levels ≥0.5ng/ml did not correlate significantly with clinical pregnancy rates. Measurement of AMH supports clinical decisions, but alone it is not a suitable predictor of IVF success [15]. However, they did not report that a relationship between the cut-off level of serum AMH and the age limit of patient undergoing on the number of embryos obtained. Their level of AMH ≤1.26ng/ml (≤4 oocytes) was higher than our S-AMH level of 0.284ng/ml (oocytes=5; sensitivity, 83.3%. specificity, 92.9%; 95% CI, 0.268 to 0.851). The Gnoth group used a high dose of IVF compared with our dose of IVF treatment, and designated two groups of women; those who were under 35 years and from 35 to 39 years. We suggest that these differences of result occur in between the Gnoth group and our study that it might be due to the doses used during ovarian stimulation and design of group. Other hand, in this study, we showed that the mean FF-AMH level was 0.639±0.290 ng/ml, and there was no relationship between the FF-AMH level and age of women undergoing IVF treatment (r=0.102, p=0.007, 95%CI; -.024 to 0.025). Lee et al. reported that FF-AMH is a marker that reflects ovarian reserve and response to controlled ovarian hyperstimulation. But, FF-AMH levels did not correlate

significantly with age, gonadotrophin dose, the number of follicles on the hCG day, and the number of oocytes retrieved [16]. It is interesting that the findings of the FF-AMH in Lee's study were different from our own; for example, the sample sizes were different between these investigations (our study vs. Lee's study; n=28 vs. n=87 patients). Second, the dosing protocols of IVF were different between these studies; Lee's study had two protocols, including the Gn-RH-a long protocol group (n=43,FF-AMH=1.8±0.4ng/ml), in which Gn-RH-a triptorelin (Decapeptyl, 0.1mg/d; Ferring, Malmo, Sweden) was started in the mid-luteal phase of the previous cycle. After pituitary down-regulation, the triptorelin dose was reduced to 0.05mg/dl, and recombinant FSH (Gonal-F; Serono, Genova, Switzerland) was added when two or more follicles reached a diameter of 17 mm, and the other protocol was the GnRH agonist multiple-dose flexible protocol (n=44, FF AMH=1.3±0.3), recombinant FSH (Gonal-F, Serono) was started on the 2nd and 3rd menstrual-cycle day without pretreatment with oral contraceptive [16]. However, our results possibility demonstrated that S-AMH levels exhibits a significant relationship between age of patient undergoing IVF and the successful developed embryos, compared with FF-AMH levels, in the form a protocol that implemented the GnRH agonist- a short protocol in this study.

4.2 3D ultrasound

The vascular indices determined by analysis of the 3D-power Doppler histogram represent the proportion of vascularized tissue (or vascularization index: VI), the amplitude of blood movement in the sampled tissue (or flow index: FI) and the proportion of vascularized and blood movement in the volumetric sample (or vascularization flow index: VFI) [17]. In a recent study, the VI, which was defined as the percentage of the power Doppler signal within a defined volume of interest, was suggested to be representative of the number of vessels in, or the vascularity of, the region of interest [18]. It is that characteristic of FI that is comprised of blood flow and attenuation factors, for example, the signal intensity is a direct consequence of the erythrocyte concentration [19], tissue motion [20], and machine settings [21]. Indeed, Dubiel et al. found in their flow phantom study that a four-fold increase in flow velocity only resulted in a 10% increase in FI. This, together with our data suggests that, despite the often-reported assumption, FI does not represent flow [22]. The 3D-power Doppler vascular indices provide a description of the amount and/or the movement of blood in a volumetric image (tissue). Therefore, vascularization and/or flow in the entire organ may not be represented by an individual tissue sample, or sampled tissues with different volumes will most likely present different vascular indices [20]. Jones et al. found using *in vitro* dual perfusion of human placental lobules, a predictable relationship between flow rates and the vascular indices, VI and VFI, however the FI was a less reliable predictor of flow; thus, it should be interpreted with caution [23]. Our results agree with the report by Jones et al. Furthermore, we explain that the results of FI are related to tissue motion and the specific techniques of each investigator. In the anatomy of the ovary, the ovarian artery, a branch of the aorta, courses along the infundibulopelvic ligament and the mesovarium border of the ovary, where it anastomoses with the ovarian branch of the uterine artery. Approximately 10 arterial branches arise from this arcade and penetrate the ovarian hilus, becoming markedly coiled and branched as they course through the medulla. These helicine arteries have longitudinal ridges of intimal smooth muscle. At the corticomedullary junction, the medullary arteries and arterioles form a plexus, from which smaller, straight

cortical arterioles arise and penetrate the cortex in a radial fashion, perpendicular to the ovarian surface. These cortical arterioles branch and anastomose several times, forming sets of interconnected vascular arcades. These arcades give rise to capillaries, which form dense networks within the theca layers of the ovarian follicles [24]. In conclusion, we automatically and quickly detected the number of follicles using Sono AVC, and vascularization around the follicles in the ovary by analysis of the 3D-power Doppler histograms. These results were expected to provide an indicator of ovarian function during IVF treatment. The S-AMH was related to the age of women undergoing COH, further investigations of the level of S-AMH which was necessary to confirm the cut-off level, and the VI might be parameter of vascularized formation around follicle in ovary using 3D-power Doppler histogram.

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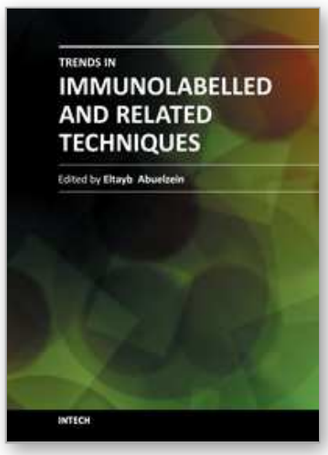

Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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