Androstenedione response to recombinant human FSH is the most valid predictor of the number of selected follicles in polycystic ovarian syndrome: (a case-control study)

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Abstract

Background: We aimed to test the hypothesis that the correlation of the changes in the blood Androstenedione (A4) levels to the number of selected follicles during ovulation induction with low-dose recombinant human follicle stimulating hormone (rhFSH) is as strong as the correlation to changes in the blood Estradiol (E2) levels in polycystic ovary syndrome (PCOS).

Methods: Prospective Case-control study conducted from October 2014 to January 2016. 61 non-PCOS control (Group I) and 46 PCOS (Group II) patients treated with the chronic low-dose step up protocosl with rhFSH. A4, E2, progesterone blood levels and follicular growth were monitored. Univariate and hierarchical multivariable analysis were performed for age, BMI, HOMA-IR, A4 and E2 (with the number of selected follicles as the dependent variable in both groups). ROC analysis was performed to define threshold values for the significant determinants of the number of selected follicles to predict cycle cancellations due to excessive ovarian response.

Results: The control group (Group I) was comprised of 61 cycles from a group of primary infertile non-PCOS patients, and the study group (Group II) of 46 cycles of PCOS patients. The analysis revealed that the strongest independent predictor of the total number of selected follicles in Group I was the E2 (AUC) (B = 0.0006[0.0003-0.001]; P < 0.001); whereas for Group II, it was the A4 (AUC) (B = 0.114[0.04-0.25]; P = 0.01). Optimum thresholds for the A4 related parameters were defined to predict excessive response within Group II were 88.7%, 3.1 ng/mL and 5.4 ng*days for the percentage increase in A4, the maximum A4 value and area under the curve values for A4, respectively.

Conclusion: A4 response to low-dose rhFSH in PCOS has a stronger association with the number of follicles selected than the E2 response. A4 response preceding the E2 response is essential for progressive follicle development. Monitoring A4 rather than E2 may be more preemptive to define the initial ovarian response and accurate titration of the rhFSH doses.

Trial registration: The study was registered as a prospective case-control study in the ClinicalTrials.gov registry with the identifier NCT02329483.

Keywords: Androgens, Androstenedione, Polycystic ovary syndrome, Ovulation induction, Folliculogenesis, Human FSH, Gonadotropins

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Background
Oligoovulation related to polycystic ovarian syndrome is treated with ovulation induction medications [1]. These patients have an increased risk of excessive ovarian response which is closely associated with the number of selected follicles [2]. Therefore, milder protocols have been developed [3]. In PCOS cases, the estradiol response to gonadotropin treatment is delayed and discordant with the visualized follicular responses [3]. Androstenedione (A₄), mostly synthesized in the ovaries, is a precursor of E₂ [4, 5].

In this study, we aimed to test the hypothesis that the cumulative changes in A₄ during ovulation induction with low dose rhFSH in PCOS cases are correlated to the number of selected follicles (follicles sized ≥12 mm) comparable to the the cumulative changes in E₂.

Methods
This is a prospective case-control study conducted between October 2014 and January 2016. Ethical permission was obtained from the Bagcilar Research and Training Hospital Research Ethics Committee. It was recorded as a prospective case-control study in the ClinicalTrials.gov registry with the identifier NCT02329483. The study was conducted in accordance with the Declaration of Helsinki. Informed consent for participation was obtained from all patients.

Study setting
The study was conducted at the Bagcilar Research and Training Hospital Gynecology and Obstetrics Department, Infertility Section. Cycle monitoring was done with folliculometry with transvaginal sonography, E₂, P₄, and A₄ measurements.

Study population
A total of 107 cycles of 61 Control-nonPCOS infertile (Group I) and 46 Study-PCOS infertile (Group II) women were included in the study. The study group was comprised of patients with anti-Müllerian hormone (AMH) levels ≥5 ng/mL (which is equivalent to PCOM (polycystic ovary morphology sonographically confirmed) [6–8].

PCOS was defined as the copresence of at least one of two of the following criteria combined with the PCOM; (1): oligoamenorrhea-OA: cycle length > 35 days, (2): hyperandrogenism (HA): presence of clinical findings including hirsutism defined as the presence of coarse [long/pigmented] terminal hair over the most commonly encountered three or more regions within the Modified Ferriman-Gallwey Score System (the buttocks/perineum, sideburn, and neck areas which contributed greatly to the score in the geographic locale where this study was conducted) with or without elevated blood testosterone levels (>0.5 ng/mL) [7–12]. The control group was comprised of unexplained primary/secondary infertile women with AMH levels <5 ng/mL, not displaying any clinical findings associated with hyperandrogenism (HA) and with regular menstrual cycles. The inclusion criteria for both groups included ages within 20-35, with normal spermograms or with mild male factor infertility (i.e.: male partners with only one the following abnormalities: sperm counts being lower than 20 million/ml or showing a normal morphology quotient of less than 4% or having a sperm motility lower than 40%; AND with post-wash total motile sperm counts equal to or higher than 5 million/ml), normal anatomic findings with the hysterosalpingography (no bilateral tubal obstruction or Müllerian anomalies), hormonally eugonadotropic, normal blood prolactin/thyroid-stimulating hormone (TSH) levels and being planned for controlled ovarian hyperstimulation and intrauterine insemination treatment. Exclusion criteria included: diabetes mellitus, BMI < 20 or >30, hypo or hypergonadotropism, other causes for hyperandrogenism, ≥2 abortions or ectopic pregnancy, additional medical disorders, ovarian cysts or previous pelvic surgery.

Controlled ovarian hyperstimulation and intrauterine insemination
Ovulation induction was conducted with follitropin alpha (Gonal-f Multidose 450 IU; Merck-Serono-Turkey) starting with a dose of 37.5 U/day or 50 U/day as described by Homburg et al. [2]. Ovulation induction was started on the 3rd or the 4th day of a menstrual cycle having early cycle blood E₂ levels ≥50 pg/mL and blood Progesterone levels <0.5 ng/mL and in the absence of any ovarian residual follicles larger than 15 mm to rule out the presence of a corpus luteum or any other cystic ovarian structure which could require further clarification. If a primary follicle response characterised by the appearance of a selected growing follicle of ≥10 mm and a rise ≥25% in blood E₂ levels was not observed despite 14 days of rhFSH stimulation, the initial dose was increased initially to 75 U/day and +37.5 U/day, weekly at each additional incremental step (i.e. 112.5, 150 U/day). Blood E₂, P₄, and A₄ levels were measured and follicle growth was monitored with transvaginal sonography at every visit (every 2-3 days) starting on day 2 or 3 of the cycles. Once 1 or 2 mature follicles ≥18 mm were observed, rhCG (Ovitrelle 150 μg; Merck-Serono-Turkey) s.c. was administered. Sperm washing and intrauterine insemination were carried within [36th–40th] hours. On the 15th day postinsemination, blood beta-hCG levels were measured and conception confirmed if the beta-hCG blood level was higher than 20mIU/mL.

Cycle cancellation policy
Cycle cancellations were due to excessive ovarian responses (more than 2 selected follicles ≥16 mm or blood E₂ level > 1500 pg/mL on the rhCG trigger day), no
ovarian response despite dose step-up and stimulation for 28-30 days, or premature luteinisation (P₄ blood level ≥ 1.3 ng/mL).

**Laboratory analysis of blood samples**

Blood samples for hormone measurement were collected from the antecubital vein with a single puncture at every visit during ovulation induction. Samples were collected in a sterile tube and transferred to the lab on the same day. All except the A₄ blood level measurement results were reported to the physicians on the afternoon of the same day. A₄ blood levels were available 7-10 days later, and did not provide any guidance to management. A colorimetric ELISA assay (Abcam-USA; Kimera Istanbul-Turkey) was used to measure A₄ levels. Measurements of AMH were made by using the AMH/MIS enzyme-linked immunosorbent assay. Testosterone values were assayed with the competitive immunoenzymatic colorimetric method. The serum FSH, luteinising hormone (LH), TSH, E₂, and prolactin levels were measured using a chemiluminescent microparticle immunoassay.

**Data analysis**

Univariate parametric tests were used for group comparisons. Significance was defined as a P-value <0.05. A₄ (AUC) was calculated as the sum of the areas of trapezoids. Primary A₄ response during ovulation induction was considered when a rise of ≥25% was observed in the basal A₄ level. HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) Index was calculated for each patient by using an online calculator.

Hierarchical multivariable regression analysis was conducted in both groups to study in a three level linear regression model the contribution of three sets of independent variables including the (Model 1) age, BMI and HOMA-IR variables; (Model 2) the E₂(AUC) values and (Model 3) (the androstenedione related variables: the primary blood androstenedione level and the A₄ (AUC) values); stepwise, defining changes in R² values (ΔR²) representing the additional effect of each of these newly added independent variable sets, on the total number of selected follicles. The SPSS 20.0 and Microsoft Excel 2010 were used.

ROC analysis was performed to define threshold values of the significant determinants of the number of selected follicles to predict cycle cancellations due to excessive ovarian response.

**Results**

A total of 107 cycles of infertile women Group I: 61 non-PCOS and Group II: 46 PCOS were followed in 16-months. The PCOS phenotypes were: PCOM (polycystic ovarian morphology; AMH ≥ 5 ng/mL)/OA (oligo/amenorrhea): 4 patients; PCOM/HA (Hyperandrogenism): 27 patients; PCOM/HA/OA: 15 patients. Mild male factor infertility was present in (22/61) 36% of Group I and (15/46) 32.6% in Group II (P = 0.5). The HOMA-IR score was ≥4.5 in (6/61) (9.8%) and (9/46) (19.6%) of patients in Groups I and II, respectively. The patient characteristics are summarised in (Table 1). There were (6/61) (9.8%) and (9/46) (19.5%) conceptions in Groups I and II, respectively. The comparison of hormonal characteristics among the conceived and the nonconceived subjects within Groups I and II are summarized in (Table 2). No conception was achieved in the absence of a primary androstenedione response earlier than the primary estradiol response. Cancelled cycles were not included in this comparison.

| Table 1 Cycles analysed in this study | Group I (Control Group) | Group II (PCOS Group) |
|--------------------------------------|-------------------------|-----------------------|
| Age                                  | 29.8 ± 0.6              | 28.7 ± 0.6            |
| Duration of infertility (years)      | 3.5 ± 0.3               | 3.4 ± 0.4             |
| BMI (kg/m²)                          | 25 ± 0.7                | 27.7 ± 0.6            |
| AMH (ng/mL)                          | 2.4 ± 0.2               | 9 ± 0.9               |
| HOMA                               | 2.8 ± 0.4               | 3.8 ± 0.4             |
| FSH (mU/mL)                          | 7.2 ± 0.3               | 6.3 ± 0.2             |
| LH (mU/mL)                           | 6.5 ± 0.5               | 9.8 ± 0.6             |
| TSH (μU/mL)                          | 2.7 ± 0.2               | 3.3 ± 0.7             |
| PRL (ng/mL)                          | 20.9 ± 1.2              | 22 ± 1.9              |
| Initial Dose (IU/day)                | 70 ± 4.1                | 65 ± 3.7              |
| Cycle Length (days)                  | 12.2 ± 4.1              | 17 ± 0.6              |
| Primary follicular/E₂ response day²  | 6.7 ± 0.4               | 9.4 ± 0.4             |
| EM at trigger day (mm)               | 9.9 ± 0.3               | 94 ± 0.3              |
| Maximum E₂ (pg/dl)                   | 453 ± 42.1              | 5569 ± 893            |
| Follicles >16 mm (n)                 | 1.1 ± 0.1               | 1.4 ± 0.2             |
| Follicles 12-16 mm (n)               | 1.2 ± 0.2               | 1.6 ± 0.3             |
| Total number of follicles            | 1.9 ± 0.2               | 2.2 ± 0.3             |
| P₄ at trigger day (ng/mL)            | 0.7 ± 0.1               | 0.8 ± 0.1             |
| Day 3 Total Testosterone (ng/mL)³   | 0.34 ± 0.28             | 0.87 ± 0.3            |
| Primary androstenedione level (ng/mL)⁴ | 0.9 ± 0.1            | 1.4 ± 0.1             |
| Primary androstenedione respond day⁵ | 6.2 ± 0.3               | 7.4 ± 0.4             |
| Time Lag (A₄ → E₂) initial responses | 0.5 ± 0.4               | 2 ± 0.5               |
| Maximum A₄ (ng/mL)⁶                  | 1.8 ± 0.1               | 2.6 ± 0.3             |
| Rise in A₄ (%)⁷                      | 66.5 ± 6.9              | 84.1 ± 7.4            |
| A₄ (AUC) (ng*days)³                  | 3.6 ± 0.3               | 5.5 ± 1.3             |
| A₄ on the trigger day (ng/mL)⁴       | 1.3 ± 0.1               | 2.5 ± 0.4             |

PCOS polycystic ovarian syndrome, AMH Anti-Mullerian hormone, HOMA Homeostasis model for assessment of insulin resistance, FSH Follicle-stimulating hormone, LH Luteinising hormone, TSH Thyroid-stimulating hormone, E₂ Estradiol, P₄ Progesterone, AUC area under the curve, A₄ Androstenedione.

²P < 0.05
Nineteen cycles were cancelled due to excessive response \((n = 8)\), no response \((n = 6)\), or premature luteinisation \((n = 5)\). In those cycles cancelled due to no response, there was no primary \(A_4\) response.

Univariate analysis revealed that the primary, maximum, and AUC values for \(A_4\) and the primary blood testosterone levels were all higher in Group II than those in Group I (Table 1).

Plateauing or decreasing \(A_4\) levels before the trigger day were observed in 33/61 (54.1%) of completed cycles in Group I and 14/46 (30.4%) of cycles in Group II. None of these made any significant difference in the basic characteristics or outcome parameters.

The correlation of \(A_4\) parameters with the selected follicle numbers and \(E_2\) (AUC) values are summarised in Figs. (1 and 2; and Tables (3 and 4): The \(A_4\)(AUC) was

Table 2 Comparison of the hormonal characteristics among the conceived and nonconceived subjects

|                      | Group I (Control Group) | Group II (PCOS Group) |
|----------------------|--------------------------|------------------------|
|                      | Did not conceive         | Conceived              | Did not conceive | Conceived |
| Primary \(A_4^a\)    | 0.88 ± 0.45              | 0.96 ± 0.2             | 1.39 ± 0.08      | 1.4 ± 0.33 |
| \(A_4\) (AUC)^b      | 3.06 ± 2.8               | 2.8 ± 1.3              | 4.2 ± 0.7        | 10.7 ± 7.1 |
| \(E_2\) (AUC)^c      | 1916.6 ± 206.3           | 2942.7 ± 1058.4        | 2654.2 ± 677.3   | 4212.7 ± 767.2 |
| AMH^d                | 2.53 ± 1.2               | 3.0 ± 0.41             | 9.23 ± 1         | 8.6 ± 1.5   |
| HOMA-IR^e            | 3.30 ± 0.3               | 1.3 ± 0.6              | 3.2 ± 0.34       | 2.22 ± 0.33 |
| Time Lag \(A_4\)-\(E_2\) Response | −0.92 ± 0.46     | −0.67 ± 0.4            | −0.23 ± 0.11^f   | −3.79 ± 0.86^f |

\(^a\)The initial blood androstenedione level (ng/mL)
\(^b\)Area under the curve value for Androstenedione (ng*days)
\(^c\)Area under the curve value for estradiol (pg*days)
\(^d\)Antimüllerian hormone (ng/mL)
\(^e\)Homeostasis Model for Assessment of Insulin Resistance
\(^f\)\(P<0.05\)
correlated with the number of selected follicles and $E_2(AUC)$ in Group II, but not correlated with any one of these parameters in Group I. The primary $A_4$ levels were correlated to the primary testosterone levels in both Groups I and II.

Hierarchical multivariable regression analysis was conducted separately for Groups I and II. The findings of the analysis are summarized in Tables 5(a,b) and 6(a,b). The total number of selected follicles ($\geq 12$ mm) was the dependent (outcome) variable. The effect of age, BMI and HOMA-IR on the total number of selected follicles were not significant in either Group I or II. In Group I: the estradiol (AUC) was the strongest independent factor ($B = 0.0006[0.0003-0.001]; P < 0.001$), whereas in Group II: the $A_4(AUC)$ ($B = 0.114 [0.04-0.25]; P = 0.01$) was the strongest independent factor affecting the total number of selected follicles.

Optimum thresholds for the $A_4$ related parameters were defined to predict excessive response within Group II.

Table 3 Correlation of $A_4$ parameters with cycle outcome parameters in Group I (Control Group)

| Parameter | $R^2$ | Primary $A_4$ | $A_4 %$b | Max $A_4$ | $A_4$ AUC |
|-----------|------|--------------|----------|-----------|-----------|
| Foll 12-16a | NS | 0.36γ | NS | NS |
| Foll >16c | NS | NS | NS | NS |
| Maximum $E_2$ | NS | 0.43γ | NS | NS |
| Total FollSelected | NS | 0.32γ | NS | NS |

$E_2 = Estradiol$, $A_4 = Androstenedione$, $AUC = Area$ under the curve;

Foll = Number of follicles

a$P < 0.005$

bPearson Correlation constant
cPercentage rise in the $A_4$ level
dnumber of follicles $> 16$ mm
eNumber of follicles $12-16$ mm

Table 4 Correlation of $A_4$ parameters to the cycle outcome parameters in Group II (PCOS Group)

| Parameter | $R^2$ | Primary $A_4$ | $A_4 %$b | Max $A_4$ | $A_4$ AUC |
|-----------|------|--------------|----------|-----------|-----------|
| Foll 12-16a | 0.513γ | 0.446γ | 0.674γ | 0.625γ |
| Foll >16c | NS | 0.3 | 0.339γ | 0.31γ |
| Maximum $E_2$ | 0.514γ | 0.512γ | 0.717γ | 0.727γ |
| Total FollSelected | 0.542γ | 0.5γ | 0.73γ | 0.673γ |

$E_2 = Estradiol$, $A_4 = Androstenedione$, $AUC = Area$ under the curve;

Foll = Number of follicles

aPearson Correlation constant
bPercentage rise in the $A_4$ level
cnumber of follicles $> 16$ mm
dnumber of follicles $12-16$ mm
eNumber of follicles $> 12$ mm

γA significant correlation; $P<0.05$
combined were as in Table 7: 88.7%, 3.1 ng/mL and 
5.4 ng*days for the percentage increase in A₄, the 
maximum A₄ value and area under the curve values for A₄, 
respectively.

**Discussion**

In this study, we observed that in PCOS, the cumulative 
A₄ response to low-dose rhFSH is a more valid measure 
of the number of selected follicles than the cumulative 
Estradiol (E₂) response [13]. The early and midfollicular 
A₄ variations are more critical determinants than the 
late follicular variations (following follicle selection), 
because drops or plateauing observed in A₄ in the late 
stages did not influence cycle outcome. The A₄ respond 
to rhFSH was earlier than that of E₂ in cycles with pro-
gressive follicular growth and conception.

In six cycles cancelled due to lack of response to 
rhFSH, there was no A₄ response. In contrast, in four of 
the eight cycles cancelled due to excessive response, a 
dosage step-up had been made due to lack of E₂ 
response, while there had already been an initial A₄ 
response. If this corrective information could have 
been taken into account, an unnecessary step-up could have 
been avoided.

PCOS is the most common cause of anovulatory infer-
tility, and is reported to comprise 15.3% of the women 
living in the geographic region where this study was 
conducted [14]. PCOS is characterised by three main 
elements: follicular growth arrest, hyperandrogenism 
and excessive folliculogenesis [15].

| Table 5 Hierarchical multivariable regression analysis of independent variables in Group I (Control Group) |
|---|
| Models | Coefficients |
| Age | 0.0004 |
| BMI | −0.02 |
| HOMA-IR | −0.19 |
| E₂(AUC) | 0.0006 |
| Primary A | 0.48 |
| A₄(AUC) | 0.01 |

Model 1: including the independent variables Age, BMI, HOMA-IR

| Table 6 Hierarchical multivariable regression analysis of independent variables in Group II (PCOS Group) |
|---|
| Models | Coefficients |
| Age | −0.06 |
| BMI | −0.12 |
| HOMA-IR | −0.08 |
| E₂(AUC) | 0.002 |
| Primary A | 0.57 |
| A₄(AUC) | 0.11 |

Model 1: including the independent variables Age, BMI, HOMA-IR

The chronic low-dose step up protocol used for 
follicular growth arrest, hyperandrogenism and excessive folliculogenesis [15].

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rhFSH is propagated to the theca cell component. During this initial response, in PCOS patients, the reversal of the FSH/LH effect in favour of FSH and aromatisation may not be as hormonally evident with rising E2 blood levels as the thecal androgen response, especially at earlier stages. Granulosa cells of the antral follicle at this stage normally respond to this early rise in A4 by increasing their aromatase activities, which is strongly counteracted by high AMH levels in the follicular microenvironment of the PCOS follicles, analogous to the AMH-FSH counteraction at later stages of follicular growth [17]. Thus, the transient follicular growth arrest is observed at this early stage due to the AMH-androgen counteraction [18, 19]. High androgen and AMH concentrations also contribute to the microenvironment that fosters excessive folliculogenesis and follicular growth arrest [11, 20–23].

Basal androgen blood levels measured at the beginning or during induction cycles have been reported in various studies in low responders, patients with diminished ovarian reserves, or normal responders, but not in PCOS cases. Ferrario et al. showed in a group of older women with low response that A4 levels measured at the beginning of IVF cycles were predictors of positive outcome [24]. Similarly, Sun et al. have shown in a study of 1413 infertile women going through their first cycle of IVF that the testosterone blood levels measured at the beginning of the treatment cycles were predictors of the number of follicles larger than 14 mm on the day of hCG trigger, but were not predictors of conception [25]. It would be interesting and supplementary to monitor the testosterone response to rhFSH during ovulation induction cycles in prospective observational studies.

Our study had some potential causes of bias and limitations which need to be addressed. From the perspective of the Rotterdam 2003 definition of PCOS, cases with the nonPCOM/HA/OA (defined as phenotype B) may be being underrepresented in the infertile PCOS patients group (Group II) [26]. Another limitation was that the BMI’s in Group II were slightly, but significantly higher than Group I. However, in the hierarchical multivariable regression conducted, BMI as well as age and HOMA-IR were included in a separate model and their effects on the total number of selected follicles were found to be insignificant in both Groups I and II.

### Conclusion

The findings in this clinical study suggest that the reactive rise in androstenedione in the early follicular phase is a better predictor of the number of follicles selected than the conventionally used reactive rise in estradiol in PCOS cases. The longer/higher is the increase in its blood levels, the more are the follicles joining the growing cohort with an increasing risk of excessive ovarian response. On the other hand, androstenedione is an earlier and more reliable marker of the initial ovarian response to gonadotropins and this earlier response may be essential for progressive follicle growth and possibly a conception in an ovulation induction and intrauterine insemination cycle (using rhFSH) in PCOS. It still needs to be further studied in prospective studies encompassing induction cycles managed mainly with A4 monitoring, to provide stronger evidence if androstenedione monitoring provides a more valid and useful information to indicate ovarian response and if an earlier androstenedione response is associated with conception.

### Abbreviations

- A4: Androstenedione; AMH: Antimüllerian hormone; AUC: Area under the curve; beta-hCG: beta human chorionic gonadotroopin; BMI: Body mass index; E2: Estradiol; FSH: Follicle stimulating hormone; HA: Hyperandrogenism; HOMA-IR: Homeostatic model assessment of insulin resistance; IVF: In vitro fertilization; OA: Oligo/amenorrhea; P4: Progesterone; PCOM: Polycystic ovarian morphology; PCOS: Polycystic ovary syndrome; rhFSH: Recombinant human follicle stimulating hormone; ROC: Receiver operating curve; TSH: Thyroid stimulating hormone

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### Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

### Authors’ contributions

SEO conceptualized and suggested a plan for the study. TY then joined the study group and helped improve the plan and theoretically better defined the study plan and its connotations of possible results. With contribution of GA the study was started. The data collection and input was carried out by SEO and GA. The data analysis and manuscript writing was done by SEO and the manuscript read and approved by the three authors.

### Competing interests

The authors declare that they have no competing interests.
**Ethics approval and consent to participate**

All women gave their informed consent. The study was reviewed and approved by the Ethics Committee of the Bagcilar Training and Research Hospital (2015/312). All patients gave informed consent for participating in this study.

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