Austin Journal of Reproductive Medicine & Infertility

Open Access

Austin Publishing Group

Research Article

Age and BMI as Major Factors Contributing to Follicular Fluid 'Oxidative/Inflammatory Biomarkers Levels and ART Outcome: A Cluster Analysis

Costa L[1,2], Oliveira P[3], Oliveira JC[4], Pires I[2], Cabral M[2], Figueiredo H[2], Felgueira E[2], Fonseca BM[1]* and Rebelo I[1]

[1]UCIBIO, REQUIMTE, Laboratório de Bioquímica, Departamento de Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Portugal

[2]Unidade de Medicina da Reprodução Dra, Ingeborg Chaves, Centro Hospitalar de Vila Nova de Gaia/Espinho E.P.E, Portugal

[3]EPIUnit - Departamento de Estudo de Populações, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

[4]Serviço de Química Clínica, Departamento de Patologia, Centro Hospitalar e Universitário do Porto, Portugal

***Corresponding author:** Fonseca BM, Faculdade de Farmácia da Universidade do Porto, Departamento de Ciências Biológicas-Laboratório de Bioquímica, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

Received: January 06, 2022; **Accepted:** February 15, 2022; **Published:** February 22, 2022

Abstract

During the last decades, due to its incidence, infertility became a focus of study. Nowadays, it is estimated to affect between 8 and 12% of reproductive-age couples worldwide. Advanced maternal age and increased body mass index (BMI) were recognized as main factors responsible for the observed trend. However, it is still not clear which mechanisms underlie such evidence and whether the two factors interact. In this work, we combined data from serum hormone levels, follicular fluid biomarkers levels, patients' intrinsic characteristics and IVF outcome from 225 patients enrolled in IVF cycles. Data were statistically analyzed, which naturally grouped patients into 4 different clusters, distinguishable by BMI and age. Here, we noted the impact of age mainly on follicular fluid biomarkers of oxidative status and of BMI on inflammation. A retrospective second analysis, based on the clusters resulted from the first one, included data from 904 IVF cycles, and the results confirmed the impact of age and obesity on IVF outcome. A logistic model revealed that unsuccess risk (defined as failure to achieve pregnancy after fresh embryo transfer) is 2.2 higher in older women (>35 years old), and 2.3 higher in obese women. There was no interaction effect between BMI and age, being the effects cumulative. Thus, although age cannot be changed, weight loss by itself may improve reproductive potential. Here, we confirm and reinforce the importance of maternal age and BMI for infertility and provide an up-to-date overview about the impact of these factors on female fertility.

Keywords: Fertility; Follicular fluid; Body Mass Index (BMI); Age; Assisted Reproductive Techniques

Abbreviations

PCOS: Polycystic Ovary Syndrome; ART: Assisted Reproductive Technologies; BMI: Body Mass Index; E2: Estradiol; LH: Luteinizing Hormone; FSH: Follicle Stimulating Hormone; AMH: Anti-Müllerian Hormone; FF: Follicular Fluid; GCs: Granulosa Cells; IVF: *In vitro* Fertilization; CCO: Corona-Cumulus-Oocyte Complexes; FET: Fresh Embryo Transfer; ICSI: Intracytoplasmic Sperm Injection; βhCG: Human Chorionic Gonadotropin; CRP: C-Reactive Protein; TAS: Total Antioxidant Status; SOD: Superoxide Dismutase; AOPPs: Advanced Oxidation Protein Products; TH: Total Hydroperoxides

Introduction

Infertility (and subfertility) is a global public health problem, estimated to affect between 8 and 12% of reproductive-aged couples worldwide [1,2], reaching almost 30% in the populations with higher prevalence [3]. The time of unwanted non-conception, female age and disease-related infertility are three major factors that influence the spontaneous conception [4,5]. Infertility can affect one or both elements of the couple [6]. Premature ovarian insufficiency/failure, polycystic ovary syndrome (PCOS), endometriosis, uterine fibroids and endometrial polyps are the most common causes of female infertility [6].

Despite enormous advances in Assisted Reproductive Technologies (ART), the success rates remain relatively low. Whereas much has been published about nonmodifiable risk factors associated with assisted reproductive outcome, such as female age and genetic factors [6,7], less attention has been devoted to modifiable behavioural risk factors that may also influence assisted reproductive outcome, such as smoking habits and Body Mass Index (BMI). Estradiol (E2), Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and, more recently, Anti-Müllerian Hormone (AMH) [8,9] are routinely measured to estimate female ovarian reserve and ovarian stimulation response, in order to program the more suitable stimulation protocol in ART. However, such measurements do not provide information about the oocyte potential to generate a good quality embryo, capable to implant and deliver a healthy new-born.

Follicular Fluid (FF) composition results from the contribution of blood plasma constituents, that cross the blood follicular barrier, and from Granulosa Cells (GCs) secretory activity [10]. Since FF provides the microenvironment for oocytes development [11-13], it represents an optimal source of non-invasive biochemical predictors of reproductive potential. Any dysregulation in FF composition can alter ovarian follicular dynamics and, thus, impair oocyte quality and fertility. Although the research in this area has progressed towards a more complex type of molecular analysis, no FF reliable biochemical predictors of oocyte quality have been determined so far, nor the

Citation: Costa L, Oliveira P, Oliveira JC, Pires I, Cabral M, Figueiredo H, et al. Age and BMI as Major Factors Contributing to Follicular Fluid 'Oxidative/Inflammatory Biomarkers Levels and ART Outcome: A Cluster Analysis. Austin J Reprod Med Infertil. 2022; 8(1): 1059.

main factors affecting FF composition.

In this work, we studied the routine parameters evaluated in the laboratory (E2, FSH, LH and AMH) together with biochemical parameters associated with antioxidant status and inflammatory response in FF. The main objective of this research study was to define groups with similar subjects, with respect to clinical and biochemical parameters by performing a cluster analysis. Therefore, we performed a statistical analysis to group similar observations into a number of clusters based on the observed values for each individual. Subsequently, i) a cluster analysis was performed using all the patient's intrinsic, plasmatic and follicular fluid variables measured and ii) based on the cluster analysis results, we evaluated the clinical significance of the findings on ART outcome. This is an observational study with a retrospective analysis of past data.

Material and Methods

Patient recruitment

This study was approved by the Ethical Committee of the Hospital (Centro Hospitalar de Vila Nova de Gaia/Espinho, E.P.E) and by the National Data Protection Commission (authorization number 526/2017).

It was conducted in two samples (Group A and Group B) of women undergoing IVF at the Human Assisted Reproduction Unit Dra. Ingeborg Chaves, Centro Hospitalar de Vila Nova de Gaia/Espinho, Portugal. Since the patients included in this study were enrolled in IVF at a Portuguese public hospital, due to the law, the maximum age allowed to be engaged in in vitro techniques was 40 years (excluded). Also, patients who underwent dual stimulation or fertility preservation cycles were excluded.

Group A consists of 225 patients enrolled in IVF cycles from March to December 2018. All female patients provided a written consent for the collection of their follicular fluid and cycle-related data, before entering the study.

Group B was used as a retrospective analysis of data belonging to 904 IVF cycles performed between January 2016 and August 2018.

As a result, there were some patients that were common to both groups A and B.

Controlled ovarian stimulation and follicular fluid sampling

Ovarian stimulation was performed accordingly to clinical evaluation. The dosage of gonadotrophins was based on the patient's age, BMI, clinical history, early follicular phase serum AMH levels and antral follicle count. Follicular maturation was accessed by serial transvaginal ultrasound scan and estradiol measurements. When the follicles reached the appropriate number and size, final maturation was induced, and oocyte retrieval was performed transvaginally under ultrasound guidance and intravenous sedation.

During oocyte aspiration, FF was collected into tubes and emptied into petri dishes. Then, the aspirated fluid was examined under a stereomicroscope and an embryologist identified, isolated, and collected the Corona-Cumulus-Oocyte complexes (CCO) for IVF. Thereafter, the remaining FF was transferred to 50mL polypropylene tubes. FF that presented obvious blood contamination was rejected. These tubes were then kept at 37°C for a maximum of 2 hours and transported to the laboratory for sample processing. FF samples were centrifuged at 300g, for 10 min at 21°C and the supernatants were filtered (using 0.45µm filters), aliquoted and kept at -80°C until further analysis.

Evaluation of fertilization, embryo quality and IVF outcome

Approximately 18h after insemination (classical IVF or Intracytoplasmic Sperm Injection - ICSI) the embryologist checked for the presence of two pronucleus, to confirm oocyte fertilization. Embryo development was evaluated accordingly to cell number, size and presence of fragments or other structures.

Embryo transfer (1 or 2 embryos) occurred on day 3 or 5 of development. For this study, the number of good quality embryos corresponded to the number of transferred plus cryopreserved embryos. When Fresh Embryo Transfer (FET) was performed, 16 days after oocyte pick-up the patient performed a blood test to measure human Chorionic Gonadotropin (βhCG) to confirm pregnancy. After additional 15 days, the presence of an embryonic sack was confirmed by echography. After birth, delivery data were also recorded.

Serum and follicular fluid measurements

Early follicular phase serum LH, FSH, E2 and AMH were measured in the hospital laboratory. Concerning follicular fluid, quantification of C-reactive protein (CRP), Total Antioxidant Status (TAS), Superoxide Dismutase (SOD) and Glutathione were performed automatically using Randox commercial kits, following manufactures' instructions. Advanced Oxidation Protein products (AOPPs) and Total Hydroperoxides (TH) were measured using in-house spectrophotometry methods.

Retrospective analysis

To explore the clinical relevance of the groups that resulted from the cluster analysis, the data from 904 cycles from January 2016 to August 2018 were collected from FileMaker Pro 4.0 database. The collected data included basal serum FSH, LH, E2 and AMH levels; mean IVF attempt; infertility time; duration of ovarian stimulation; number of collected oocytes; percentage of mature (MII) oocytes; fertilization rate; number of two-pronuclear (2PN) zygotes; percentage of FET; reason for no FET; number of transferred embryos; implantation rate per FET; percentage of live birth per FET; number of newborns; weeks of gestation; and birth weight.

Statistical analysis

Cluster Analysis was carried so that groups with similar subjects, with respect to the study variables, could be defined. The different clusters were compared by an Analysis of Variance (ANOVA), and when the assumptions of normality or homogeneity of variance were not observed, by the non-parametric test of Kruskal-Wallis. Comparisons between groups were based on the Tukey HSD test or on the non-parametric Mann-Whitney test. In the case of categorical variables, Chi-square or Fisher´s exact test were used. A logistic regression model was developed taking as response variable the unsuccessful of the fertility treatment and independent variables age and BMI in classes. All analysis was carried out in IBM SPSS Statistic 25. Significance was assessed for $p<0.05$.

Results

Group A: cluster analysis

The mean age of the studied population was 34.84 (3.46). 2.2% of women presented low BMI, 64.9% had normal BMI, 24.9% were overweight and 8% were obese. The reason for fertility treatment was exclusively female subfertility in 27.5% of the couples and 27.8% male-only infertility. A total of 17.5% suffered from an idiopathic unexplained infertility cause. There was no significant difference in success rates between IVF and ICSI treatments.

A total number of 225 IVF cycles were grouped into clusters using three sets of variables: i) BMI and age; ii) fertilization rate, percentage of mature oocytes, percentage of obtained quality embryos per oocyte; iii) all clinical and biochemical parameters. First, a hierarchical cluster analysis was performed for each set of variables to determine the number of clusters to be considered. From the analysis of the dendrograms, a number of clusters between 3 and 5 seemed to capture the different assemblage groups. Subsequently, for each set, a k-means cluster analysis was performed, using 3, 4 and 5 centroids. From this approach, it was observed that, when using the set with the variables BMI and age, the results were very similar to those obtained with the set using all variables. The clusters obtained based on the 3 variables that we considered to reflect the success of the treatment (fertilization rate, percentage of mature oocytes and percentage of obtained quality embryos per oocyte) did not lead to groups that distinguished themselves with respect to age and BMI, as well as to the variables measured in the follicular fluid.

The groups obtained considering two (BMI and age) and five variables (BMI, age, fertilization rate, percentage of mature oocytes and percentage of obtained quality embryos per oocyte) led to similar results regarding serum hormone measurements and treatment success variables. Therefore, we concluded that the two determining variables in the definition of clusters were BMI and age. Considering the importance of these two variables (BMI and age) on cluster definition, three sets of clusters were considered: patients grouped in three, four and five clusters. The different cluster arrangements were compared with respect to the observed biochemical parameters.

We observed that the analysis with three and four clusters produced nearly/almost identical results in serum measurements, with a significant difference for FSH using three clusters, and for FSH and AMH using four clusters. The same differences were observed using five clusters. Regarding follicular fluid measurements, the grouping into three or four clusters showed significant differences for AOPP, TAS and CRP. Due to these findings, we kept four clusters. In addition, the division into four clusters seems to have a natural meaning, distinguishing between a) higher and lower BMI and b) young and older women. Taking into account the classification in

Table 1: Data for each cluster in Group A. Differences between groups are defined with "≠". [*Mean (standard deviation); ⊥ Median (P25-P75). Body Mass Index (BMI); Estradiol (E2); Luteinizing Hormone (LH); Follicle Stimulating Hormone (FSH); Anti-Müllerian Hormone (AMH); Fresh Embryo Transfer (FET); C-Reactive Protein (CRP); Total Antioxidant Status (TAS); Advanced Oxidation Protein products (AOPPs); Total Hydroperoxides (TH); Thyroid Stimulating Hormone (TSH)].

	Cluster 1 (C1) (-/-)	Cluster 2 (C2) (-/+)	Cluster 3 (C3) (+/-)	Cluster 4 (C4) (+/+)	Total
n (%)	65 (28.9)	101 (44.9)	28 (12.4)	31 (13.8)	225
Age (years)*	31.6 (2-0)	37.1 (1.6)	30.8 (2.5)	37.9 (1.4)	34.8 (3.5)
BMI (kg/m^2)*	21.5 (1.8)	22.4 (2.2)	28.0 (2.3)	30.4 (3.1)	24.0 (3.9)
basal FSH mIU/mL ^ (F(3,173)=3.282, p=0.022)	6.3 (5.4-7.6)	7.6 (6.1-9.7)	6.5 (5.0-7.5)	7.4 (5.5-8.1)	6.9 (5.7-8.5) **C1≠C2**
basal LH mIU/mL ^ (c^2 3)=4.481, p=0.214)	5.5 (4.1-6.9)	6 (4.3-8.0)	6.2 (4.8-8.0)	4.6 (2.7-7.0)	5.8 (4.1-7.3)
basal E2 mIU/mL ^ (F(3,147)=0.748, p=0.525)	38.4 (27.0-67.0)	50.5 (37.1-76.3)	43.3 (26.7-57.0)	38 (24.0-51.8)	43.3 (30.9-68.6)
basal AMH pmol/L ^ (c^2 3)=15.691, p=0.001)	21.5 (9.4-36.2)	13.1 (5.5-24.4)	29 (10.0-51.0)	9.9 (4.5-19.7)	16.9 (6.8-30.6) **C4¹C1;C4¹C3 C2¹C1;C2¹C3**
TSH ^ (F(3,101)=0.130, p=0.942)	1.7 (1.4-2.3)	1.6 (1.1-2.5)	1.4 (0.8-2.2)	1.7 (1.0-2.7)	1.7 (1.1-2.3)
TAS mmol/L ^ (F(3,146)=4.023, p=0.009)	1.05 (1.0-1.3)	0.9 (0.8-1.1)	1.1 (0.9-1.3)	1 (0.9-1.3)	1.0 (0.9-1.2) **C1¹C2**
AOPP ^ µM of chloramine-T equivalent (F(3,189)=3.537, p=0.016)	232.5 (155.9-302.5)	188.6 (145.4-263.6)	244.4 (197.5-303.6)	208.6 (112.0-260.9)	208.6 (146.1-274.8) **C3¹C4**
TH µm/g protein ^ (c^2 3)=6.981, p=0.073)	0.5 (0.3-0.6)	0.4 (0.3-0.6)	0.4 (0.3-0.4)	0.5 (0.4-0.8)	0.4 (0.3-0.6)
FOX1 µM ^ (F(3,189)=2.182, p=0.092)	30.9 (26.4-39.9)	27.1 (21.8-35.7)	30.3 (25.3-36.2)	32.7 (27.5-40.5)	29.8 (23.8-37.2)
CRP mg/L ^ (c^2 3)=31.437, p<0.001)	0.7 (0.3-2.0)	1 (0.2-1.9)	2.5 (1.2-4.4)	3.2 (1.1-7.4)	1.2 (0.3-2.7) **C1¹C3;C1¹C4 C2¹C3;C2¹C4**
Retrieved oocytes^ (c^2 3)=19.135, p<0.001)	11 (6.3-15.0)	6 (3.0-10.8)	10.5 (6.0-16.8)	7 (3.8-12.3)	8 (4-13) **C1¹C4;C1¹C2 C3¹C2**
% of MII oocytes ^ (F(3,214)=0.186, p=0.906)	80 (60.0-90.0)	80 (60.0-100.0)	80 (70.0-90.0)	80 (60.0-100.0)	80 (64.0-100.0)
% fertilization^ (F(3,208)=0.949, p=0.418)	75 (50.0-92.0)	67 (50.0-88.5)	63 (36.5-75.0)	58.5 (33.0-96.5)	67 (45.3-66.8)
Total number of good quality embryos ^	2 (1-5)	2 (1-2.5)	1 (2-2)	2 (0.8-2.3)	2 (1-3)
Cycles with FET	49	80	22	23	174
% of FET	75.4	79.2	78.6	74.2	77.3
% of implantation (per FET) (c^2 6)=9.925, p<0.0019)	61.2	38.8	40.9	21.7	43.1

Table 2: Data for each cluster in Group B. Differences between groups are defined with "≠". [*Mean (standard deviation); ⊥ Median (P25-P75). Body Mass Index (BMI); Estradiol (E2); Luteinizing Hormone (LH); Follicle Stimulating Hormone (FSH); Anti-Müllerian Hormone (AMH); Fresh Embryo Transfer (FET)].

	Cluster 1 (C1) (-/-)	Cluster 2 (C2) (-/+)	Cluster 3 (C3) (+/-)	Cluster 4 (C4) (+/+)	Total
n (%)	282 (31.2)	409 (45.2)	111 (12.3)	102 (11.3)	904
Age (years)*	31.5 (2.2)	37.4 (1.5)	30.9 (2.3)	37.4 (1.7)	34.7 (3.5)
BMI (kg/m^2)*	21.7 (1.7)	22.5 (2.1)	27.7 (2.0)	30.9 (2.6)	23.8 (3.7)
basal FSH mIU/mL ^ (c^2 3)=16.5, p=0.001)	6.6 (5.7-7.9)	7.2 (5.8-9.7)	6.4 (5.2-7.5)	6.7 (5.4-7.9)	6.8 (5.5-8.4) **$C3^1C2$; $C4^1C2$; $C1^1C2$**
basal LH mIU/mL ^ (c^2 3)=16.5, p=0.001)	5.0 (4.0-7.0)	5.0 (3.0-7.0)	5.0 (4.0-6.0)	4.0 (3.0-5.0)	5.0 (4.0-7.0) **$C4^1C2$; $C4^1C3$; $C4^1C1$**
basal E2 mIU/mL ^ (c^2 3)=12.2, p=0.007)	43.0 (31.0-61.0)	40.0 (6.5-59.8)	39.0 (29.0-57.0)	37.0 (27.3-53.8)	41.0 (25.0-58.8) **$C2^1C1$**
basal AMH pmol/L^ (c^2 3)=61.5, p<0.001)	21.1 (11.9-35.9)	10.0 (4.2-22.3)	21.0 (9.1-48.6)	13.1 (5.1-29.4)	15.2 (5.9-29.9) **$C2^1C1$; $C2^1C3$; $C4^1C1$; $C4^1C3$**
Retrieved oocytes^ (c^2 3)=33.1, p<0.001)	9.0 (5.0-14.0)	6.0 (3.0-10.0)	7.0 (4.0-13.0)	7.0 (3.0-12.3)	7.0 (3.0-29.9) **$C2^1C3$; $C2^1C1$; $C4^1C1$;**
% of MII oocytes^ (c^2 3)=1.9, p=0.583)	80.0 (67.0-93.0)	81.8 (66.7-100.0)	80.0 (67.0-91.0)	79.5 (64.0-97.0)	80.0 (66.7- 100)
% fertilization^ (c^2 3)=4.3, p=0.231)	60.0 (39.5-81.5)	65.6 (39.6-87.5)	60.0 (33.0-75.8)	59.0 (40.0-79.8)	60.0 (38.0-83.0)
Total number of good	2.0 (1.0-3.0)	2.0 (1.0-2.0)	1.0 (1.0-2.0)	2.0 (0.75-2.0)	2.0 (1.0-2.0)
quality embryos^ (c^2 3)=4.2, p=0.240)	(0-12)	(0-12)	(0-11)	(0-9)	
Cycles with FET	205	281	82	71	639
% of FET	72.7	68.7	73.9	69.6	639/904 = 70.7
Implanted embryos % of implantation (per FET) (c^2 3)=19.5, p<0.003)	51.2	37	43.9	29.6	266/639 (41.6)
% of delivery (per FET) (c^2 3)=26.4, p<0.001)	46.3	26.7	37.8	21.1	217/639 (36.3)
Gestational Weeks * (F(3,210)=1.1, p=0.356)	38.1 (2.0)	38.4 (1.9)	37.6 (2.8)	38.1 (2.0)	38.1 (2.1)
% of unsuccess (no delivery after FET)	110/205 (53.7)	206/281 (73.3)	50/82 (61.0)	56/71 (78.9)	422/639 (66.0)

four clusters, patients are distinguished with the symbols "+" and "-" corresponding to a higher (+) / lower (-) BMI and to an older (+) or younger (-) age.

The main characteristics and patient distribution between clusters of group A sample are presented in Table 1. Concerning serum variables, a significant difference was found in AMH values between cluster 4 for clusters 1 and 3 and between cluster 2 for clusters 1 and 3. As expected, it seems that age is the factor that justifies this difference in AMH levels, with cluster 4 being the one with the highest value. For FSH, we also found significant differences between groups. Although cluster 4 presents a higher FSH level, there is only a significant difference between cluster 1 and 2. Thus, as in the case of AMH, age seems to be the differentiating factor. For E2 and LH serum variables, no significant differences were found between clusters.

Regarding follicular fluid measurements, significant differences were found for AOPPs, TAS and CRP. For Glutathione, SOD and TH, no differences were found. For AOPPs a significant difference was found between cluster 3 and 4. Both clusters include patients with higher BMI. Thus, once again, age seems to justify the difference. For TAS, there was a significant difference between cluster 1 and 2. Among women with low BMI, the youngest present high TAS values. In the case of CRP, the analysis revealed a significant difference between cluster 1 to clusters 3 and 4 and between cluster 2 to clusters 3 and 4. Patients from clusters 1 and 2 have lower BMI. Thus, while age is associated with TAS and AOPS, BMI is associated with follicular fluid CRP levels.

Concerning IVF outcome, the number of retrieved oocytes is significantly different between cluster 1 to clusters 4 and 2 and between cluster 3 and 2. Age is apparently responsible for this difference, since younger women retrieve a greater number of oocytes. However, no significative difference was found for fertilization rate or number of good quality embryos.

Table 3: Logistic Regression model (BMI - Body Mass Index).

	n (%)	Odds Ratio (95% Confidence Interval)
Age (25-29)	61 (9.5)	1
Age (30-34)	227 (35.5)	1.025 (0.579-1.814)
Age (>=35)	351 (54.9)	2.206 (1.259-3.867)
BMI (<=25)	454 (71.0)	1
BMI (25-30)	135 (21.1)	1.088 (0.721-1.641)
BMI (>=30)	50 (7.8)	2.277 (1.069-4.849)

Group B: Retrospective analysis

To investigate the impact of BMI and age on ART outcome, we performed a retrospective analysis of 904 cycles from January 2016 to August 2018. The analysis performed for Group A was replicated for Group B and patients were divided according to BMI and age, also forming four clusters.

The majority (45.2%) of patients belongs to cluster 2 (older, low BMI) (Table 2). As in the analysis of Group A, this cluster presents higher serum FSH levels (compared to all other clusters) and lower AMH levels (versus clusters 1 and 3). In contrast, younger patients with low BMI (cluster 1) present lesser IVF attempts and infertility time, while presenting the highest rate of elective single embryo

transfer (eSET) (data not shown) and implantation rate per transfer, as well as the highest birth rate per fresh embryo transfer (FET). These patients also have the highest percentage of no FET due to indication for freeze all (to prevent hyperstimulation syndrome – OHSS). In contrast with the first analysis, Group B study revealed significant differences for LH between cluster 4 and all the other clusters; and for E2 between cluster 2 and 1. Concerning the number of retrieved oocytes, patients from cluster 1 present the highest value. The differences between clusters of the group B sample are described in Table 2. The percentage of mature oocytes does not appear to be impacted by BMI and age nor by the fertilization rate and weeks of gestation.

Implantation and delivery rate, the most important indicators of ART success, differ between clusters, though not significantly. Unsuccess, defined by no live birth after FET, as expected, is higher in cluster 4 and lower in cluster 1.

Logistic regression model the delivery of a newborn after fertility treatment can be seen as the main outcome which may depend on age and BMI. Therefore, a logistic regression model (Table 3) was developed considering as independent variables age and BMI of the patients grouped into three classes (age: 25-29; 30-34; >=35 and BMI: <=25; 25-30; >=30). The reference classes for age and BMI were, 25-29 years and BMI less or equal to 25kg/m^2, respectively.

The logistic model shows that, with respect to age, the risk of an unsuccessful fertility treatment measured by the odds ratio, increases with age, being significantly greater (2.2 times) for the older age class with respect to the younger class. Concerning BMI, the risk increases, with women in the last class (BMI>=35) presenting an odds ratio (2.3) significantly greater when compared to the women in the lowest BMI class.

There is no interaction effect between age and BMI. It should be noted that most of the women are in the last age class.

Discussion and Conclusion

Although several studies have already demonstrated that BMI and age have a great impact on female fertility [7,14], most authors looked for direct correlations between the measured markers and/or IVF outcome and BMI/age. In our work, we combined data from serum hormone levels, follicular fluid biomarkers levels, patients' intrinsic characteristics and IVF outcome. The data were then analyzed, and patients naturally grouped into four different clusters that further presented a natural meaning, distinguishing between normal/higher BMI and young/older women.

From both analysis (Group A and B) a significant difference was found for AMH between younger and older patients. As expected, younger women have higher AMH levels. Nowadays, serum AMH is consistently used as a biomarker of ovarian reserve [9,15,16], being considered by the European Society of Human Reproduction and Embryology (ESHRE) reliable biomarker for the prediction of ovarian response categories [17]. Moreover, follicular fluid-AMH (and progesterone) from individual follicles have revealed a potential use for predetermining subsequent embryonic developmental competence [13].

Additionally, FSH also significantly differs with age. Although FSH is still used to estimate patients' response to gonadotrophin stimulation, it is currently falling into disuse. These biomarker is currently considered by ESHRE not sufficiently reliable to predict ovarian response [17]. In the analysis of Group A, and concerning serum hormone levels, no significant differences between clusters were found for E2 nor LH. However, with a larger sample (Group B), significant differences were found for the two variables. LH presents lower values in the older and heavier patients, which attend progressive age-associated decrease and obesity-related disturbances in serum gonadotropins levels [18,19]. Previous studies have shown that serum E2 concentrations are directly associated with BMI [20]. Although in both groups the number of retrieved oocytes was higher in younger patients, it did not reflect on fertilization rates or number of good quality embryos. However, our data show a higher number of good quality embryos obtained from younger patients with low BMI and, more importantly, a higher implantation and delivery rate.

Regarding the performed follicular fluid measurements, we selected markers that may reflect the oxidative and inflammatory status of intra follicular microenvironment. A disruption of the oxidative homeostasis may result in oxidative stress, which has already been described to interfere with female fertility and reproduction, mainly by affecting ovarian folliculogenesis, steroidogenesis, oocyte maturation, ovulation and luteolysis and, consequently, embryo development and reproductive success [21]. No significant differences between clusters were found for Glutathione, SOD and TH. In contrast, younger patients present higher AOPPs and TAS concentrations in FF. Also in blood plasma, elevated antioxidant status was suggested to favour IVF/ICSI followed pregnancy [22]. However, the effect of antioxidants on female fertility is still not clear and no reliable main effect has been detected so far. Nevertheless, AOPPs were already suggested as a potential biomarker to predict oocyte quality and outcome of IVF in infertile women with endometriosis [23].

Inflammation is a well-known and common thread in cardiovascular disease, arthritis and immune disorders, and new evidence also points as a factor in infertility [24,25]. If inflammation is chronic, it may disturb reproductive physiology, leading to fertility problems. In fact, many fertility problems are linked to inflammatory processes and immune system imbalance [25]. It can affect ovulation and hormone production as well as being associated with endometriosis [26,27]. We found that BMI is directly associated with follicular fluid CRP levels. This observation is in line with previous studies, that showed that CRP in FF raises with increasing BMI [28]. Serum CRP levels also appear to negatively affect embryo quality [29]. Several studies have already reported the detrimental consequences of obesity on female fertility. It has been associated with deregulated menstrual cycle [30,31], increased infertility time [32], lower oocyte quality [27], increased risk of miscarriage [33,34] and gestational diabetes [35]. However, there is no conclusive evidence that modifiable factors such as BMI, have a negative effect on ART outcome, suggesting that the effect of obesity on oocyte quality and fertility outcome is complex and multifactorial.

In our work we further performed a retrospective analysis, with a larger group of patients, to investigate the clinical relevance of our findings. In this approach, we confirmed the negative impact of high BMI and advanced age on fertility but, unlike other studies, we did not correlate age or BMI directly with IVF outcome. Instead, we suggest

that different combinations of age and BMI intervals differently affect IVF outcome and should be carefully evaluated. Moreover, the logistic model revealed that the unsuccess risk (defined by failure to achieve pregnancy after fresh embryo transfer) is 2.2 higher in women after 35 years old compared to the youngest class (25-29 years old). Similarly, obese women present a 2.3 higher risk of unsuccess compared with women with normal BMI. As no interaction effect was found between BMI and age, the effects are cumulative. In fact, the weight loss in overweight and obese women may improve fertility and sometimes be sufficient to restore fertility and get pregnant spontaneously [36,37]. Thus, despite age cannot be changed, there are modifiable factors such as BMI that have a great impact in fertility.

ART professionals should strongly recommend weight loss in overweight and obese patients before including them in ART programs, in order to optimize the results. This study also highlights the important role of nutritional counselling when caring for overweight patients who plan to conceive [37]. More detailed studies should be performed to understand which factors may be affected by age and BMI that could justify the impact on female fertility, in order to help physicians to plan the most appropriate stimulation protocols and develop better treatments for infertile women.

Declaration

Authorship: Lia Costa contributed to the study design, the acquisition, analysis and interpretation of data, drafted the manuscript, and approved the final version to be published. Pedro Oliveira contributed to the study design, analysis, and interpretation of data, and revised and approved the final version to be published. José Carlos Oliveira, Ilda Pires, Madalena Cabral, Helena Figueiredo and Eduarda Felgueira contributed to acquisition of data and approved the final version to be published. Bruno Fonseca and Irene Rebelo contributed to the study design, interpretation of data, and revised and approved the final version to be published.

Funding: This work was financial supported by PTDC/MEC-OUT/28931/2017.

Acknowledgments: This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia in the framework of the project POCI-01-0145-FEDER-028931 BiokART; and by Lia's PhD grant PD/BD/128334/2017 from FCT PhD Programme in Medicines and Pharmaceutical Innovation (i3DU).

References

1. Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. Human reproduction update. 2008; 14: 605-621.
2. Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Human reproduction update. 2015; 21: 411-426.
3. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS medicine. 2012; 9: e1001356.
4. Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges J, Freundl G. Definition and prevalence of subfertility and infertility. Human reproduction (Oxford, England). 2005; 20: 1144-1147.
5. Jose-Miller AB, Boyden JW, Frey KA. Infertility. American family physician. 2007; 75: 849-856.
6. Vander Borght M, Wyns C. Fertility and infertility: Definition and epidemiology. Clinical biochemistry. 2018; 62: 2-10.
7. Crawford NM, Steiner AZ. Age-related infertility. Obstetrics and gynecology clinics of North America. 2015; 42: 15-25.
8. Medicine PCotASfR. Testing and interpreting measures of ovarian reserve: a committee opinion. Fertility and sterility. 2015; 103: e9-e17.
9. Jamil Z, Fatima SS, Ahmed K, Malik R. Anti-Mullerian Hormone: Above and Beyond Conventional Ovarian Reserve Markers. Disease markers. 2016; 2016: 5246217.
10. Siu MK, Cheng CY. The blood-follicle barrier (BFB) in disease and in ovarian function. Advances in experimental medicine and biology. 2012; 763: 186-192.
11. Yoo SW, Bolbot T, Koulova A, Sneeringer R, Humm K, Dagon Y, et al. Complement factors are secreted in human follicular fluid by granulosa cells and are possible oocyte maturation factors. The journal of obstetrics and gynaecology research. 2013; 39: 522-527.
12. Tan J, Zou Y, Wu XW, Tian LF, Su Q, He JX, et al. Increased SCF in Follicular Fluid and Granulosa Cells Positively Correlates With Oocyte Maturation, Fertilization, and Embryo Quality in Humans. Reproductive sciences (Thousand Oaks, Calif). 2017; 24: 1544-1550.
13. O'Brien Y, Wingfield M, O'Shea LC. Anti-Mullerian hormone and progesterone levels in human follicular fluid are predictors of embryonic development. 2019; 17: 47.
14. Imterat M, Agarwal A, Esteves SC, Meyer J, Harlev A. Impact of Body Mass Index on female fertility and ART outcomes. Panminerva medica. 2019; 61: 58-67.
15. Bhide P, Pundir J, Homburg R, Acharya G. Biomarkers of ovarian reserve in childhood and adolescence: A systematic review. 2019; 98: 563-572.
16. Khan HL, Bhatti S. Antral follicle count (AFC) and serum anti-Mullerian hormone (AMH) are the predictors of natural fecundability have similar trends irrespective of fertility status and menstrual characteristics among fertile and infertile women below the age of 40 years. 2019; 17: 20.
17. Group EREG. Guideline on Ovarian Stimulation for IVF/ICSI. 2019.
18. van Zonneveld P, Scheffer GJ, Broekmans FJ, Blankenstein MA, de Jong FH, Looman CW, et al. Do cycle disturbances explain the age-related decline of female fertility? Cycle characteristics of women aged over 40 years compared with a reference population of young women. Human reproduction (Oxford, England). 2003; 18: 495-501.
19. Beydoun HA, Beydoun MA, Wiggins N, Stadtmauer L. Relationship of obesity-related disturbances with LH/FSH ratio among post-menopausal women in the United States. Maturitas. 2012; 71: 55-61.
20. Rinaldi S, Key TJ, Peeters PH, Lahmann PH, Lukanova A, Dossus L, et al. Anthropometric measures, endogenous sex steroids and breast cancer risk in postmenopausal women: a study within the EPIC cohort. International journal of cancer. 2006; 118: 2832-2839.
21. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reproductive biology and endocrinology: RB&E. 2012; 10: 49.
22. Velthut A, Zilmer M, Zilmer K, Kaart T, Karro H, Salumets A. Elevated blood plasma antioxidant status is favourable for achieving IVF/ICSI pregnancy. Reproductive biomedicine online. 2013; 26: 345-352.
23. Song Y, Liu J, Qiu Z, Chen D, Luo C, Liu X, et al. Advanced oxidation protein products from the follicular microenvironment and their role in infertile women with endometriosis. Experimental and therapeutic medicine. 2018; 15: 479-486.
24. Niringiyumukiza JD, Cai H, Xiang W. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. Reproductive biology and endocrinology: RB&E. 2018; 16:

43.

25. Vannuccini S, Clifton VL, Fraser IS, Taylor HS, Critchley H, Giudice LC, et al. Infertility and reproductive disorders: impact of hormonal and inflammatory mechanisms on pregnancy outcome. Human reproduction update. 2016; 22: 104-115.

26. Weiss G, Goldsmith LT, Taylor RN, Bellet D, Taylor HS. Inflammation in reproductive disorders. Reproductive sciences (Thousand Oaks, Calif). 2009; 16: 216-229.

27. Snider AP, Wood JR. Obesity Induces Ovarian Inflammation and Reduces Oocyte Quality. Reproduction (Cambridge, England). 2019.

28. Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, et al. Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. The Journal of clinical endocrinology and metabolism. 2009; 94: 1533-1540.

29. Haikin Herzberger E, Miller N, Ghetler Y, Tamir Yaniv R, Neumark E, Shulman A, et al. A prospective study of C-reactive protein in patients with obesity during IVF. Human fertility (Cambridge, England). 2019: 1-6.

30. Douchi T, Kuwahata R, Yamamoto S, Oki T, Yamasaki H, Nagata Y. Relationship of upper body obesity to menstrual disorders. Acta obstetricia et gynecologica Scandinavica. 2002; 81: 147-150.

31. Lash MM, Armstrong A. Impact of obesity on women's health. Fertility and sterility. 2009; 91: 1712-1716.

32. Brewer CJ, Balen AH. The adverse effects of obesity on conception and implantation. Reproduction (Cambridge, England). 2010; 140: 347-364.

33. Boots C, Stephenson MD. Does obesity increase the risk of miscarriage in spontaneous conception: a systematic review. Seminars in reproductive medicine. 2011; 29: 507-513.

34. Cavalcante MB, Sarno M, Peixoto AB, Araujo Junior E, Barini R. Obesity and recurrent miscarriage: A systematic review and meta-analysis. The journal of obstetrics and gynaecology research. 2019; 45: 30-38.

35. Ogburn PL. Obesity and gestational diabetes in pregnancy: an evolving epidemic. Journal of perinatal medicine. 2016; 44: 361-362.

36. Clark AM, Thornley B, Tomlinson L, Galletley C, Norman RJ. Weight loss in obese infertile women results in improvement in reproductive outcome for all forms of fertility treatment. Human reproduction (Oxford, England). 1998; 13: 1502-1505.

37. Kort JD, Winget C, Kim SH, Lathi RB. A retrospective cohort study to evaluate the impact of meaningful weight loss on fertility outcomes in an overweight population with infertility. Fertility and sterility. 2014; 101: 1400-1403.