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ASSISTED REPRODUCTION TECHNOLOGIES

The cumulative dose of gonadotropins used for controlled ovarian stimulation does not influence the odds of embryonic aneuploidy in patients with normal ovarian response

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Abstract
Objective Controlled ovarian hyperstimulation (COH) promotes multifollicular growth, increasing the chance of obtaining euploid embryos that will successfully implant. Whether aneuploidy is increased from COH with exogenous gonadotropins interfering with natural selection of dominant follicles is a concern. This study evaluates the association between gonadotropin exposure and aneuploidy.
Methods This is a retrospective cohort study of 828 patients that underwent 1122 IVF cycles involving controlled ovarian stimulation and trophectoderm biopsy for preimplantation genetic screening (PGS), from 2010 to 2015. Polymerase chain reaction (PCR) was used to assess aneuploidy. Kruskal-Wallis tests and logistic regression with generalized estimating equations (GEEs) were used for data analysis.
Results Overall, after controlling for patient age, ovarian reserve, stimulation protocol, days of stimulation, and diagnoses, there was no significant association between cumulative gonadotropin (GND) dose and the odds of aneuploidy (adjusted OR = 1.049, $p = 0.232$). Similarly, in cycles where patients did not require COH beyond cycle day 12, there was no significant association between cumulative gonadotropin dose and the odds of aneuploidy (adjusted OR = 0.909, $p = 0.148$). However, in cases where patients were stimulated past cycle day 12, there was a significant increase in the odds of aneuploidy (adjusted OR = 1.20, 95% CI 1.125–1.282, $p < 0.0001$) with increasing cumulative gonadotropin dose, with a small effect size (Cohen's $d = 0.10$, 95% CI 0.08–0.12). In this cohort, there was a 16.4% increase in the odds of aneuploidy for each 1000-u increase in cumulative GND exposure (adjusted OR = 1.164, $p = 0.002$). When the analysis was restricted to low responders (peak estradiol <500 pg/mL or <4 mature follicles achieved; there was no significant association between gonadotropin dose and aneuploidy (adjusted OR = 1.12, 95% CI 0.982–1.28, $p = 0.09$), regardless of the duration of COH required to reach vaginal oocyte retrieval.
Conclusion The degree of exposure to exogenous gonadotropins did not significantly modify the likelihood of aneuploidy in patients with a normal ovarian response to stimulation (not requiring COH beyond cycle day 12). Patients requiring prolonged COH were demonstrated to have elevated odds of aneuploidy with increasing cumulative gonadotropin dose. This finding may reflect an increased tendency towards oocyte and embryonic aneuploidy in patients with a diminished response to gonadotropin stimulation.

Keywords Aneuploidy · Human embryos · Controlled ovarian stimulation · In vitro fertilization · Preimplantation genetic screening · Exogenous gonadotropins

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Introduction

The aim of controlled ovarian hyperstimulation (COH) with exogenous gonadotropins (follicle-stimulating hormone (FSH) and/or luteinizing hormone (LH)) is to maximize the number of oocytes yielded in an effort to overcome the high

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rate of attrition of gametes and embryos during IVF treatment. Embryonic aneuploidy is the leading cause of poor oocyte quality, embryonic arrest, implantation failure, and spontaneous early pregnancy loss [1, 2]. While advanced maternal age is most often implicated, ovarian stimulation of suboptimal follicles, containing poorer quality oocytes that would have otherwise been naturally selected to undergo atresia, has been hypothesized to lead to an increase in oocyte aneuploidy. COH has been proposed to influence oocyte maturation and the completion of meiosis, potentially mediating chromosomal aneuploidy and mosaicism [3]. Based on these theories, an increasing number of fertility centers recently reverted to using minimal stimulation protocols, despite a lack of evidence-based, peer-reviewed data to support their efficacy [4]. Determining whether the dose of exogenous gonadotropins used for COH influences the incidence of embryonic aneuploidy is necessary to optimize ovarian stimulation.

The use of preimplantation genetic screening (PGS) to selectively transfer euploid embryos has been shown to improve implantation and clinical pregnancy rates while reducing the incidence of early pregnancy loss [5]. PGS, using recent, clinically validated technology such as polymerase chain reaction (PCR), is now commonly used to determine the ploidy status of trophectoderm cells obtained from blastocyst biopsy. While a few early animal studies support an association between gonadotropin stimulation and embryonic aneuploidy [6], there is a lack of consensus in the human studies using PGS. Previous investigators have reported that the proportion of aneuploid embryos in patients was reduced after mild stimulation protocols or natural cycles [7–9]. However, Verpoest et al. [10] demonstrated an unexpectedly high rate of embryonic aneuploidy in embryos derived from unstimulated cycles (36.4%). All prior human studies investigating the effect of COH on embryonic ploidy involved screening a limited number of chromosomes, using fluorescent in situ hybridization (FISH) analysis of blastomeres from day 3 embryos. PGS with FISH is now known to have suboptimal diagnostic accuracy due to its operator-dependent nature, hybridization failure, and signal overlap and has been shown to have a deleterious effect on clinical outcome [11]. Over the past decade, major technological advances in molecular biology have increased the accuracy and precision of PGS and allowed for an assessment of genetic competence in blastocysts, rather than cleavage stage embryos. Studies utilizing more recent PGS methods are needed to provide an accurate assessment of whether gonadotropin dose influences the development of aneuploidy. The purpose of this study is to explore the relationship between the cumulative dose and duration of gonadotropin stimulation and the incidence of embryonic aneuploidy in blastocysts derived from IVF cycles, in which a more recent, validated PGS technology was used.

Materials and methods

This single-center retrospective cohort analysis included infertility patients who completed an IVF cycle with PGS from March 2010 to April 2015. Patients aged 18 to 45 years, who underwent COH and had their blastocysts screened for aneuploidy by PGS with quantitative PCR, were identified from an electronic medical record database and included in the study. Oocyte donor recipients were excluded. Research approval was obtained from Western Institutional Review Board, and all subjects provided informed consent.

Ovarian stimulation

Patients underwent conventional COH for IVF. The COH protocol used was selected at the discretion of the reproductive endocrinologist and involved the administration of FSH and human menopausal gonadotropin (hMG) with a gonadotropin-releasing hormone (GnRH) agonist downregulation protocol with leuprolide acetate (Lupron®, AbbVie Inc., North Chicago, IL), a GnRH antagonist protocol (Ganirelix Acetate®, Organon USA Inc., Roseland, NJ or Cetrotide®, EMD Serono, Rockland, MA) (which could be combined with the administration of 100 mg clomiphene citrate on cycle days 3 to 7 or estrogen priming using an estradiol patch in the luteal phase of the preceding cycle), or a microflare protocol (Lupron®, AbbVie Inc., North Chicago, IL). These protocols have been described previously [12–16]. The first day of COH medication administration was denoted cycle day 3. For microflare cycles, a microdose of GnRH agonist administration commenced on day 3 with the addition of exogenous gonadotropins from day 4 onwards. Exogenous gonadotropin stimulation was initiated and dosed according to baseline ovarian reserve testing (basal antral follicle count (BAFC) by transvaginal sonogram, day 3 serum FSH, and antimullerian hormone (AMH) levels). Gonadotropin dosage was titrated according to the ovarian response to stimulation, assessed by serum estradiol rise and follicular growth on serial ultrasound scans. When two or more follicles measured greater than 18 mm in diameter, final oocyte maturation was induced with recombinant human chorionic gonadotropin (hCG) alone (Ovidrel®, EMD Serono, Rockland, MA) or in patients with high ovarian response and/or risk of OHSS undergoing a GnRH antagonist protocol, with 40 IU of leuprolide acetate (Lupron®, AbbVie Laboratories, Chicago, IL) concomitant with 1000–1500 IU of hCG (Novarel®, Ferring Pharmaceuticals, Parsippany, NJ). Vaginal oocyte retrieval (VOR) was performed 36 h later, under transvaginal ultrasound guidance.

Lab procedures

All metaphase II oocytes underwent intracytoplasmic sperm injection (ICSI), which is utilized in all PGS cycles to avoid contamination by extraneous DNA. Fertilization was assessed approximately 18 h later, and fertilized oocytes with two pronuclei were cultured to the cleavage stage in Sage Quinn's Advantage® Cleavage Medium (Cooper Surgical, Trumbull, CT) from day 0 to day 3. Media supplementation consisted of 5% human serum albumin with 100 mg/mL (HSA-Solution™, Vitrolife, Göteborg, Sweden) on day 0 and 10% of synthetic serum substitute (SSS) with 6% protein components consisting of 84% pharmaceutical grade hSA (50 mg/mL) (SSS, Irvine Scientific, Santa Ana, CA) from day 1 to 6 of development. Low-oxygen conditions were maintained from day 1 to 3 under 5% oxygen, 5.5% carbon dioxide, and 89.5% nitrogen and from day 3 to 6 under 5% oxygen, 6% carbon dioxide, and 89% nitrogen, provided by solid-state, ultra-stable, mini-incubators (Panasonic Sterisonic GxP incubator, Sanyo North America, Wood Dale, IL) using Nunclon 60-mm dishes with ten microdrops of 50 μL drops for up to one embryo per drop under 100% paraffin oil (Ovooil™, Vitrolife, Göteborg, Sweden). On day 3 after fertilization, the embryos were transferred from Sage Quinn's Advantage® Cleavage Medium (zero glucose, pyruvate-dominant) to (glucose-rich) G-2.5™ Vitrolife Blastocyst Media (Göteborg, Sweden) and supplement protein (10% SSS, Irvine Scientific, Santa Ana, CA). On day 3 of embryo development, all the embryos underwent assisted hatching, where a small 25–30-μm opening was created in the zona pellucida with a Zilostk laser (Hamilton Thorne Biosciences, Beverly, MA) to promote herniation of the trophectoderm.

On day 5, blastocysts with a herniating trophectoderm underwent biopsy. If the trophectoderm remained well contained within the zona, the embryo was cultured for another 8–24 h and reassessed. Biopsies were conducted under oil in Falcon 1006 Petri dishes (Becton Dickinson, Franklin Lakes, NJ) in 10 μL drops of Enhance WG—Vitrolife HTF/HEPES. With an Olympus IX70 microscope equipped with Narishige micromanipulators (East Meadow, NY), the blastocyst was secured with the protruding trophectoderm at the 3 o'clock position. An estimated four to seven trophectoderm cells were drawn into the lumen of a sharp, thin-walled biopsy pipette with an internal diameter of 30 μm and pulled gently away from the blastocyst. Trophectoderm cell detachment was achieved with 500 μs of near-infrared laser pulsations. The detached trophectoderm cells were processed for 24-chromosome aneuploidy screening by quantitative PCR. With either technique, the biopsied embryos were washed in blastocyst medium and transferred to individually numbered 10 μL droplets under oil; they were checked 1 day after the biopsy or at completion of the analysis for evidence of reexpansion, indicative of continuing viability.

Outcome measures

The primary objective was to analyze the relationship between cumulative gonadotropin dose used and the duration of COH with embryonic aneuploidy. The influence of maternal age, number of oocytes retrieved, and the duration of gonadotropin stimulation on aneuploidy rate was determined. The effect of total gonadotropin dose on aneuploidy rate was first examined in the context of all patients undergoing ovarian stimulation regardless of specific protocol, controlling for maternal age. Subsequent analyses were performed in patients that were stratified by the number of days of stimulation (<12 vs. ≥12 days), IVF protocol type, and cumulative gonadotropin dose quartiles.

Statistical methods

Krusal-Wallis tests were used to compare the distributions of demographic and cycle characteristics between protocols, stimulation duration (COH lasting <cycle day 12 vs. ≥cycle day 12), and cumulative gonadotropin dose quartiles. Logistic regression fit with generalized estimating equations (GEEs) were used to model the relationship between the odds of aneuploidy and the cumulative gonadotropin dose and duration of COH, while accounting for within-patient correlation of responses. All odds ratios were adjusted by controlling for age, day 3 FSH, BAFC, stimulation protocol (agonist/antagonist), days of stimulation, and diagnoses (uterine factor, diminished ovarian reserve, anovulation, hypothalamic amenorrhea, tubal factor, male factor, and endometriosis). When patients were grouped according to cumulative gonadotropin dose quartiles, the odds of aneuploidy were expressed in relation to the first gonadotropin dose quartile, as a reference. The odds of aneuploidy for individual patients were assumed to be equally correlated across cycles, which corresponded to utilizing an exchangeable working correlation structure. Odds ratio's (OR) with 95% confidence intervals and corresponding p values are presented. Hypothesis testing was performed using two-tailed tests at the alpha = 0.05 level of significance. All analyses were done in R version 3.2.4 (R Core Team 2016), using the geepack package [17]. Cohen's d was used to describe the effect sizes, using the rules of $|d| < 0.2$, 0.2–0.5, 0.5–0.8, and >0.8 as "small," "medium," "large," and "very large" effects, respectively. Continuous demographic and IVF cycle characteristics are presented as means with standard deviations.

Results

A total of 828 patients underwent 1122 IVF cycles with controlled ovarian stimulation yielding blastocysts that underwent trophectoderm biopsy. The 1122 cycles were completed in the

following distribution: 824 GnRH antagonist, 122 estrogen priming, 13 clomiphene citrate-GnRH antagonist, 38 GnRH agonist downregulation, and 125 microflare cycles (458 and 664 cycles corresponded to patients that underwent COH until before or beyond cycle day 12, respectively). Subjects who underwent gonadotropin stimulation had a median age of 38.5 years (range 35.2–40.7) and basal FSH levels of 6.3 m IU/mL (range 4.2–7.9). The baseline demographics of subjects and IVF characteristics stratified by type of protocol, duration of ovarian stimulation, and cumulative gonadotropin dose quartiles are shown in Tables 1, 2, and 3, respectively.

IVF cycles were stratified according to the ovarian stimulation protocol used. The patients within each IVF protocol group differed significantly by age ($p < 0.001$), AMH ($p < 0.0001$), day 3 FSH ($p < 0.0001$), peak estradiol levels ($p = 0.04$), basal antral follicle count (BAFC) ($p < 0.0001$), cumulative gonadotropin dose ($p < 0.0001$), duration of stimulation ($p < 0.0001$), total eggs retrieved ($p < 0.0001$), mature eggs retrieved ($p < 0.0001$), number of blastocysts biopsied ($p < 0.0001$), and aneuploidy rate ($p = 0.03$). IVF protocol groups were similar according to BMI ($p = 0.89$) and fertilization rate ($p = 0.12$). The patients receiving the protocols usually reserved for low responders (clomiphene citrate-GnRH antagonist, estrogen priming protocol, and microflare) had the lowest mean number of blastocysts biopsied ($p < 0.0001$) and the highest rates of aneuploidy ($p = 0.03$) (Table 1).

When patients were stratified according to the duration of gonadotropin stimulation (ovulation triggered before cycle day 12 vs. on cycle day 12 or later) (Table 2), patients in the longer stimulation duration cohort had a significantly increased patient age ($p < 0.01$) and BMI ($p < 0.01$), decreased BAFC ($p < 0.0001$), decreased AMH ($p < 0.0001$), increased cumulative gonadotropin dose ($p < 0.0001$), decreased number of eggs retrieved ($p < 0.0001$), decreased number of mature eggs retrieved ($p < 0.0001$), and blastocysts biopsied ($p < 0.0001$). The groups were similar in peak E2 ($p = 0.08$), fertilization rate ($p = 0.23$), and aneuploidy rate ($p = 0.43$). When patients were stratified into quartiles by the cumulative gonadotropin dose received, the quartile that received the greatest dose had the most advanced age ($p < 0.0001$), the lowest BAFC ($p < 0.0001$), the least oocytes retrieved ($p < 0.0001$), and blastocysts biopsied ($p < 0.0001$). There was a significant preponderance of patients with diminished ovarian reserve in the highest cumulative gonadotropin dose quartile ($p < 0.001$) and anovulatory patients in the lowest gonadotropin dose quartile ($p < 0.001$). The IVF protocols most often used for patients in the highest gonadotropin dose quartile were clomiphene citrate-GnRH antagonist ($p < 0.05$), estrogen priming protocol ($p < 0.001$), and microflare ($p < 0.001$) (Table 3).

Overall, aneuploidy was detected in 47% of all of the embryos resulting from 1122 IVF cycles. A univariate analysis, not controlling for modifiers or confounding factors, revealed that for each year increase in age, the odds of aneuploidy increased by 14% (OR 1.14, 95% CI 1.12–1.16; $d = 0.072$, 95% CI 0.066–0.078, $p < 0.001$). For each additional egg retrieved, the odds of aneuploidy decreased by 3% (OR 0.97, 95% CI 0.96–0.98; $d = -0.017$, 95% CI −0.020–−0.013, $p < 0.001$). For each 1000-u increase in cumulative gonadotropin dose, the odds of aneuploidy increased by 28% (OR 1.28, 95% CI 1.2–1.3; $d = 0.14$, 95% CI 0.11–0.16,

Table 1 Demographic and IVF cycle characteristics of patients stratified by IVF protocol

	GnRH antagonist	Estrogen priming/ GnRH antagonist	Clomiphene citrate/ GnRH antagonist	GnRH agonist downregulation	Microflare	*p* value
Sample size	824	122	13	38	125	–
Age	37.31 (4.22)	39.83 (3.16)	40.18 (4.06)	36.45 (3.41)	38.93 (3.18)	<0.0001
BMI	23.23 (4.16)	23.22 (4.49)	23.83 (5.02)	23.26 (4.95)	23.2 (3.86)	0.89
AMH	3 (3.47)	1.51 (1.48)	1.14 (1.52)	2.51 (1.27)	1.3 (1.23)	<0.0001
BAFC	12.2 (6.23)	8.79 (4.51)	10.65 (12.16)	13.03 (6.27)	7.85 (3.77)	<0.0001
D3 FSH	6.54 (3.24)	3.74 (2.35)	6.46 (3.49)	5.13 (2.45)	6.12 (4.02)	<0.0001
Parity	0.2 (0.46)	0.15 (0.36)	0.08 (0.28)	0.34 (0.58)	0.14 (0.4)	0.13
Gravidity	0.5 (0.9)	0.35 (0.62)	0.77 (0.73)	0.63 (0.79)	0.46 (0.69)	0.08
Cumulative GND dose	3568.07 (1333.9)	5176.46 (977.14)	4470.85 (1494.04)	2748.3 (1220.82)	4467.79 (943.62)	<0.0001
Days of stimulation	11.82 (1.42)	13.2 (1.46)	12.15 (1.82)	11.34 (1.15)	13.03 (1.82)	<0.0001
Peak E2	1666.17 (1130.38)	1594.75 (1291)	2057.23 (1476.46)	2152.71 (959.01)	1677.57 (1157.35)	0.04
Eggs retrieved	15.99 (9.32)	11.05 (6.72)	7.85 (4.74)	20.03 (9.62)	9.83 (5.4)	<0.0001
Mature eggs retrieved	12.46 (7.91)	8.55 (5.46)	6.08 (3.8)	15.58 (9.56)	7.34 (4.26)	<0.0001
Fertilization rate*	0.78 (0.18)	0.77 (0.18)	0.82 (0.21)	0.79 (0.19)	0.74 (0.21)	0.12
Blastocysts biopsied	4.67 (3.96)	2.8 (2.25)	2.62 (2.43)	5 (3.11)	2.36 (1.38)	<0.0001
Aneuploidy rate*	0.49 (0.35)	0.56 (0.41)	0.7 (0.36)	0.45 (0.33)	0.56 (0.41)	0.03

Standard deviations are shown in parentheses. Results are expressed as mean with standard deviation in parentheses and frequencies*

Table 2 Demographic and IVF cycle characteristics of patients stratified by duration of COH (stimulation until <cycle day 12 vs. ≥cycle day 12)

	All	<Cycle day 12	≥Cycle day 12	*p* value
Sample size	1122	458	664	–
Age	37.77 (4.09)	37.24 (4.4)	38.13 (3.82)	<0.01
BMI	23.23 (4.19)	22.87 (4.12)	23.48 (4.23)	<0.01
AMH	2.56 (3.1)	3.22 (4.04)	2.16 (2.25)	<0.0001
BAFC	11.36 (6.15)	12.99 (6.9)	10.23 (5.29)	<0.0001
D3 FSH	6.14 (3.35)	6.93 (2.61)	5.59 (3.68)	<0.0001
Parity	0.19 (0.44)	0.21 (0.47)	0.18 (0.43)	0.24
Gravidity	0.49 (0.85)	0.47 (0.89)	0.5 (0.81)	0.15
Cumulative GND dose	3825.89 (1385.07)	2934.96 (976.86)	4440.42 (1288.17)	<0.0001
Days of stimulation	12.1 (1.57)	10.68 (0.51)	13.07 (1.29)	<0.0001
Peak E2	1680.69 (1153.26)	1745.36 (1141.34)	1636.07 (1160.16)	0.08
Eggs retrieved	14.81 (9.05)	16.52 (10.01)	13.62 (8.13)	<0.0001
Mature eggs retrieved	11.5 (7.67)	12.73 (8.42)	10.65 (6.98)	<0.0001
Fertilization rate*	0.78 (0.18)	0.78 (0.18)	0.77 (0.19)	0.23
Blastocysts biopsied	4.2 (3.67)	4.89 (4.13)	3.72 (3.22)	<0.0001
Aneuploidy rate*	0.51 (0.37)	0.52 (0.35)	0.5 (0.38)	0.43

Results are expressed as mean with standard deviation in parentheses and frequencies*

$p < 0.001$). Lastly, for each additional cycle day of stimulation, the odds of aneuploidy increased by 7% (OR 1.07, 95% CI 1.01–1.12; $d = 0.04$, 95% CI 0.02–0.06, $p = 0.017$).

After controlling for patient age, day 3 FSH, BAFC, stimulation protocol, days of stimulation, and diagnosis, there was no significant association between cumulative gonadotropin dose and the odds of aneuploidy (adjusted OR = 1.049, $p = 0.232$). The correlation between cumulative gonadotropin dose and aneuploidy was found to be significantly modified by the duration of COH, based on a significant interaction term between gonadotropin dose and the number of days of COH ($p < 0.05$). Therefore, further analyses were stratified by the duration of stimulation using cycle day 12 as a threshold, as this was the median cycle day on which COH ended. In patients who did not require COH beyond cycle day 12, there was no significant association between cumulative gonadotropin dose and the odds of aneuploidy (adjusted OR = 0.909, $p = 0.148$). However, in patients stimulated past cycle day 12, there was a significant increase in the odds of aneuploidy (adjusted OR = 1.20, 95% CI 1.125–1.282, $p < 0.0001$) with a small effect size (Cohen's $d = 0.10$, 95% CI 0.08–0.12). In this cohort, there was a 16.4% increase in the odds of aneuploidy for each 1000-u increase in cumulative GND exposure (adjusted OR = 1.164, $p = 0.002$). In patients stimulated beyond cycle day 12, the odds of aneuploidy were higher in cycles during which patients were exposed to the third and fourth quartiles of cumulative GND dose compared to the first quartile (Q_3 vs. Q_1 adjusted OR = 1.462, $p = 0.019$; Q_4 vs. Q_1 adjusted OR = 1.828, $p = 0.002$). However, there was no significant difference in the odds of aneuploidy in cycles during which patients were exposed to the second quartile of cumulative GND dose vs. the first quartile (Q_2 vs. Q_1 adjusted OR = 1.265, $p = 0.166$) (Table 4).

In a subset of patients classified as "low responders" (635 cycles; 200 patients), defined by peak estrogen <500 pg/mL or <4 mature follicles achieved, the odds of aneuploidy increased by 18% for each year increase in age (OR 1.18, 95% CI 1.11–1.25; $d = 0.09$, 95% CI 0.07–0.11, $p < 0.001$). Controlling for age, there was no evidence of an association between gonadotropin dose and aneuploidy (adjusted OR 1.12, 95% CI 0.982–1.28; $d = 0.03$, 95% CI −0.01–0.07, $p = 0.092$). Similarly, there was no evidence of an association between duration of stimulation and aneuploidy when controlling for age and cumulative gonadotropin dose (adjusted OR 0.84, 95% CI 0.586–1.21; $d = -0.10$, 95% CI −0.17–−0.02, $p = 0.344$).

When subjects were grouped according to the ovarian stimulation protocol used, there was no significant relationship between gonadotropin dose and aneuploidy in patients who underwent stimulation that ended before cycle day 12 (antagonist, adjusted OR = 1.0, 95% CI 0.901–1.108, $d = 0.0$, 95% CI −0.03–0.03, $p = 0.98$; estrogen priming, adjusted OR = 1.049, 95% CI 0.796–1.383, $d = 0.03$, 95% CI −0.05–0.10, $p = 0.733$; clomiphene citrate-GnRH antagonist, adjusted OR = 1.717, 95% CI 0.893–3.303, $d = 0.30$, 95% CI 0.05–0.55, $p = 0.1$; GnRH agonist downregulation, adjusted OR = 1.522, 95% CI 0.845–2.74, $d = 0.23$, 95% CI 0.03–0.44, $p = 0.161$; microflare, adjusted OR = 0.569, 95% CI 0.133–2.435, $d = -0.31$, 95% CI −0.44–−0.18, $p = 0.45$). However, there was a significant correlation between gonadotropin dose and the odds of aneuploidy in patients who underwent stimulation past cycle day 12 with the GnRH antagonist (adjusted OR = 1.19, 95% CI 1.103–1.293; $d = 0.10$,

Table 3 Mean baseline demographics and IVF cycle characteristics of patients stratified by GND dose quartile

	850–2780	2780–3980	3980–4720	4720–9050	*p* value
Sample size	289	277	275	281	–
Age	35.38 (4.39)	37.52 (4.02)	39.09 (3.41)	39.18 (3.17)	<0.0001
BMI	22.68 (3.66)	23.14 (3.99)	23.61 (4.57)	23.52 (4.47)	0.21
AMH	5.43 (5.33)	2.52 (2.19)	1.87 (1.37)	1.38 (1.3)	<0.0001
BAFC	16.37 (7.42)	11.23 (5.28)	9.75 (3.88)	7.9 (3.48)	<0.0001
D3 FSH	6.12 (2.53)	6.55 (3.19)	6.92 (3.44)	4.99 (3.8)	<0.0001
Parity	0.2 (0.43)	0.16 (0.41)	0.23 (0.51)	0.17 (0.41)	0.35
Gravidity	0.5 (0.86)	0.41 (0.79)	0.59 (0.97)	0.46 (0.74)	0.18
Diminished ovarian reserve	3.46%	12.64%	18.55%	29.18%	<0.001
Uterine factor	2.08%	3.25%	4.36%	2.49%	0.42
Anovulation	13.49%	7.22%	5.09%	2.14%	<0.001
Hypothalamic amenorrhea	1.73%	1.44%	1.82%	4.27%	0.13
Tubal factor	4.15%	5.05%	4.36%	3.2%	0.74
Recurrent pregnancy loss	7.61%	4.69%	8.73%	11.03%	<0.05
Male factor	11.07%	8.3%	6.18%	5.69%	0.08
Endometriosis	1.04%	3.25%	1.45%	0.71%	0.10
Cumulative GND dose	2051.84 (485.97)	3387.49 (351.1)	4371.17 (220.34)	5548.97 (747.29)	<0.0001
GnRH antagonist/estrogen priming	0.69%	2.53%	11.64%	28.83%	<0.001
GnRH antagonist	91%	76.9%	75.64%	49.82%	<0.001
Clomiphene citrate/GnRH antagonist	0.35%	0.36%	2.55%	1.42%	<0.05
GnRH agonist downregulation	7.61%	2.53%	2.18%	1.07%	<0.001
Microflare	0.35%	17.69%	8%	18.86%	<0.001
Days of stimulation	11.13 (1.1)	11.45 (1.2)	11.85 (0.82)	13.95 (1.3)	<0.0001
Peak E2	1871.35 (985.42)	1716.82 (1113.14)	1532.55 (1181.87)	1593.95 (1292.2)	<0.0001
Eggs retrieved	21.7 (10.93)	14.81 (7.9)	12.17 (6.03)	10.29 (5.63)	<0.0001
Mature eggs retrieved	17.27 (9.64)	11.52 (6.61)	8.92 (4.69)	8.07 (4.74)	<0.0001
Fertilization rate*	0.8 (0.17)	0.77 (0.18)	0.77 (0.18)	0.77 (0.2)	0.04
Blastocysts biopsied	6.67 (4.84)	4.19 (3.45)	3.22 (2.28)	2.62 (1.78)	<0.0001
Aneuploidy rate*	0.44 (0.29)	0.5 (0.37)	0.56 (0.38)	0.54 (0.41)	<0.001

Results are expressed as mean with standard deviation in parentheses, frequencies*, and proportions (%)

95% CI 0.07–0.13, $p < 0.0001$), estrogen priming (adjusted OR = 1.27, 95% CI 1.097–1.472; $d = 0.13$, 95% CI 0.08–0.19, $p = 0.001$), and GnRH agonist downregulation (adjusted OR = 1.73, 95% CI 1.046–2.857; $d = 0.30$, 95% CI 0.09–0.51, $p = 0.033$) protocols.

Discussion

Oocyte-derived embryonic aneuploidy is the major driver of reproductive failure. COH aims to overcome human reproductive inefficiency by maximizing oocyte yield to increase the

Table 4 Adjusted odds ratios for the association between cumulative gonadotropin dose quartiles and odds of aneuploidy, stratified by duration of COH

	COH < cycle day 12	*p* value	COH ≥ cycle day 12	*p* value
Quartile 1 (850–2780)	Reference category	–	Reference category	–
Quartile 2 (2780–3980)	0.839 (0.646, 1.09)	0.188	1.265 (0.907, 1.765)	0.166
Quartile 3 (3980–4720)	0.647 (0.447, 0.938)	0.022	1.462 (1.065, 2.006)	0.019
Quartile 4 (4720–9050)	1.14 (0.696, 1.865)	0.603	1.828 (1.252, 2.669)	0.002

Odds ratios are adjusted for age, ovarian reserve, IVF protocol, and infertility diagnoses

likelihood of obtaining a euploid embryo. High doses of exogenous gonadotropins administered over an extended duration have been theorized to interfere with natural selection, increasing the selection of oocytes that do not carry a normal haploid number of chromosomes. This is the largest study, to date, to investigate whether cumulative gonadotropin dose has an effect on incidence of embryonic aneuploidy.

Analyses were stratified by the duration of stimulation using cycle day 12 as a threshold, as this was the median cycle day on which COH ended. Patients requiring COH beyond cycle day 12 to achieve criteria for oocyte retrieval were considered to have a diminished ovarian response to gonadotropins, whereas those patients who did not require extended COH were considered "normal responders." This study demonstrated that blastocyst aneuploidy was not significantly influenced by the cumulative dose of exogenous gonadotropins administered in normal responders. However, in patients who required COH beyond cycle day 12, there was a significant, dose-dependent relationship between aneuploidy and exogenous gonadotropin dose. While the length of time that COH is required to achieve adequate follicular maturity has yet to be incorporated in any established criteria to classify patients as having a normal vs. poor response to gonadotropin stimulation, it is well known that the need for COH past cycle day 12 is indicative of an underlying predisposition towards poor oocyte quantity and quality, parameters that are often seen in women of advanced age and correlated with decreased pregnancy rate [18]. This study corroborated that patients who required COH past cycle day 12 had significantly increased age, decreased oocyte yield, and decreased blastocysts available for trophectoderm biopsy. Furthermore, patients receiving the protocols usually reserved for low responders (clomiphene citrate-GnRH antagonist, estrogen priming protocol, and microflare) had significantly higher rates of aneuploidy. The increased tendency towards poor oocyte reserve and quality in patients requiring an extended duration of COH was controlled for by performing a subanalysis restricted to low responders only. Controlling for age, no association between embryonic aneuploidy and cumulative gonadotropin dose was observed in low-responder patients, regardless of COH duration.

This study's findings refute past theories which have suggested various mechanisms linking exposure to exogenous gonadotropins for COH with embryonic aneuploidy. Concern regarding the potential of exogenous gonadotropins to induce oocyte and embryonic aneuploidy first arose from studies in which young, fertile oocyte donors were reported to have a higher than expected prevalence of embryonic aneuploidy [19, 20]. High serum estradiol levels and oocyte yield have been linked to increased multinucleation of blastomeres [21] and increased chromosomal abnormalities. In addition to producing high estradiol levels, the large cohorts of follicles in high responders tend to develop in an asynchronous manner, which may require ovulation to be triggered with HCG when cytoplasmic maturity has not yet been attained in a large subset of developing oocytes [19]. Oocytes from high responders without polycystic ovarian syndrome (PCOS) have been demonstrated to have cytogenetic features suggestive of cytoplasmic immaturity, which could increase their susceptibility to errors in chromosomal segregation [22]. Mice exposed to high-dose gonadotropins were reported to have an increase rate of chromosomal aberrations; reduced blastulation; and increased embryo degeneration, triploidy, and sister chromatid exchange [23]. Conversely, other animal studies have demonstrated a lack of difference in the incidence of non-disjunction in mouse oocytes obtained after ovarian stimulation and spontaneous ovulation [24].

Unfortunately, the human studies to date provide an even greater degree of conflicting results and contradictory conclusions, possibly owing to major differences in study design and the technology used to assess embryonic ploidy. It has been reported that the degree of gonadotropin stimulation in a GnRH agonist downregulation protocol was significantly associated with granulosa cell aneuploidy, measured by flow cytometric analysis, in a dose-dependent manner [25, 26]. However, Kaleli et al. [26] reported no correlation between serum and intrafollicular estradiol levels and the rate of aneuploidy, suggesting that granulosa cell aneuploidy may have existed at baseline, prior to exogenous gonadotropin exposure. Katz-Jaffe et al. [27] performed FISH and single-cell allelic profiling to determine the degree and source of chromosome 21 aneuploidy and mosaicism in cleavage stage embryos from COH with exogenous FSH. After controlling for maternal age, the authors reported that the mean daily FSH dose that produced embryos with normal chromosome 21 division (251.6 IU) was significantly lower than the mean dose that yielded embryos with mosaic and non-mosaic mitotic (394 IU) and meiotic (363.1 IU) chromosome 21 segregation errors ($p < 0.01$). Rubio et al. [28] performed a crossover study in which 22 oocyte donors underwent COH in a GnRH agonist downregulation protocol with high FSH dose (225 IU) in an initial cycle, followed by low FSH dose (150 IU) in a subsequent cycle, at least 3 months later. Oocytes from the low-dose group had significantly increased fertilization rates and yielded cleavage stage embryos with a decreased rate of aneuploidy, determined by FISH. However, the low- and high-dose FSH groups had similar implantation and pregnancy rates [28]. Baart et al. [7] performed one of the few randomized control trials evaluating whether the mild COH strategy could reduce the incidence of aneuploidy and improve overall clinical outcome. After randomizing patients to either mild COH in a GnRH antagonist regimen or conventional COH in a GnRH agonist downregulation protocol, and using FISH to assess aneuploidy in cleavage stage embryos, the authors reported similar absolute numbers of euploid embryos but a significantly higher proportion of aneuploidy

embryos in the conventional COH cohort, suggesting that the surplus of oocytes and embryos obtained from conventional stimulation were of lower quality [7].

In agreement with the findings of this study, there are several recent investigations that utilized FISH, an earlier technology only capable of ascertaining copy number for a limited number of chromosomes, and failed to show a relationship between COH and aneuploidy in day 3 embryos [29–31]. Verpoest et al. [29] reported an aneuploidy rate of 36.4% in cleavage stage embryos from 11 unstimulated cycles, demonstrating that numerical chromosomal abnormalities are present to a significant degree in oocytes from younger women, even in the absence of COH. Labarta et al. [30] showed that cleavage stage embryos from oocyte donors undergoing COH and unstimulated cycles had similar rates of aneuploidy (40.6 vs. 34.8%, RR 1.17 [95% CI 0.8–1.8], $p = 0.45$) and implantation (32.5 vs. 39.3%, $p = 0.68$), with an increase in the cumulative live birth rate in the COH cohort (45.7 vs. 13%, $p = 0.001$). Braga et al. [31] demonstrated that the FSH dose for COH had no impact on the incidence of aneuploidy in 440 day 3 embryos from 119 cycles in infertile women aged over 38 years. Interestingly, when the authors controlled for patient age and FSH dose, they found a significant association between high oocyte yield and aneuploidy [31]. This lends credence to the theory that patients prone to high ovarian response and oocyte yield, such as those with PCOS, may have poor oocyte quality at baseline, possibly related to incomplete cytoplasmic maturation of their oocytes.

In recent years, "mild" stimulation protocols employing lower doses of exogenous gonadotropins have proposed, premised on the theory that this approach may improve pregnancy rates by minimizing embryonic aneuploidy. Other putative benefits of "minimalist" COH have included a reduction in the risk of ovarian hyperstimulation syndrome (OHSS), multiple pregnancies, and wastage of supernumerary embryos [32]. However, these concerns have been effectively countered with the increased utilization of GnRH agonist to trigger ovulation and improvements in vitrification technology, allowing for single, thawed embryo transfer in a subsequent synthetic cycle [33]. It is well established that low-dose COH leads to a marked reduction in oocyte yield, with a resulting decrease in cumulative clinical pregnancy rate [7]. In light of this, COH protocols designed to lower oocyte yield can only be justified if this reduction is offset by improved embryo quality with a lower incidence of oocyte and embryonic aneuploidy. Therefore, a study using more recent, clinically validated PGS technology to accurately assess ploidy in blastocysts is warranted to reliably investigate whether the incidence of aneuploidy is influenced by cumulative gonadotropin dose.

This is the only study to use a recent, clinically validated PGS technique to assess the rate of embryonic aneuploidy in relation to cumulative gonadotropin dose for COH. PCR has been validated by several studies to have superior diagnostic ability and clinical outcome compared to FISH [34, 35]. While many prior studies have focused on the impact of gonadotropin dose on aneuploidy in embryos derived from young, fertile, high responding oocyte donors, this study's analysis was performed using data generated from a large pool of infertility patients with a wide range of infertility diagnoses, while controlling for confounding factors such as patient age, ovarian reserve, and poor responder status. Furthermore, the use of generalized estimating equations allowed for an equal weighting of data from all patients; many of which underwent multiple IVF cycles with PGS. Therefore, these findings can be generalized to the infertile population, for whom identifying strategies to maximize oocyte and embryo quality and selection is of utmost importance.

Given its retrospective nature, this study's design is vulnerable to potential selection bias with regards to patient characteristics, gonadotropin dosing, and/or the COH protocol used. However, the statistical analyses controlled for patient age, cumulative gonadotropin dose, and the duration of COH. The assessment of embryo quality was limited to evaluation of genetic competence at the blastocyst stage. Therefore, effects of cumulative gonadotropin dose on the quality or ploidy of oocytes which failed to fertilize or embryos which arrested prior to reaching the blastocyst stage could not be studied. Furthermore, extrachromosomal factors mediating clinical outcome could not be accounted for, as the analysis was restricted to preimplantation embryos that may or may not have undergone subsequent embryo transfer. Future studies shall assess the effect of cumulative gonadotropin dose for COH on euploid embryonic competence in the form of implantation, clinical pregnancy, and live birth rates.

Although PCR is markedly superior to FISH for the accurate diagnosis of aneuploidy, PGS of cells from a trophectoderm biopsy involves the analysis of only a small fraction of the cells that make up the whole embryo and thus may underreport the incidence of embryonic mosaicism. In the future, the development of non-invasive methods of screening for embryonic aneuploidy (i.e., analysis of the embryonic secretome) may allow for a more comprehensive assessment of ploidy. This study did not attempt to determine the degree of blastocyst mosaicism. However, based on the mechanisms theorized in prior studies, any contribution of gonadotropin exposure to aneuploidy would arise from meiotic error within oocytes, which is most likely to cause aneuploidy involving the whole embryo. Based on this, mosaicism can be assumed to affect embryos from unstimulated cycles to a similar degree.

In this study, the measured cumulative gonadotropin dose took into account the combined effect of both exogenous FSH and LH. Follicular growth and oocyte maturation is a dynamic process requiring synergistic interaction of both FSH and LH. Prior studies have suggested that there may be an appropriate LH concentration threshold for euploidy and successful

implantation, as LH has been proposed to play a role in the resumption of meiosis [36, 37]. While this study's analysis did not account for the relative contributions of LH and FSH to cumulative gonadotropin dose, when IVF cycles were stratified by COH protocol, there was lack of association between embryonic aneuploidy and cumulative gonadotropin dose in COH not extending past cycle day 12. A relationship between aneuploidy and cumulative gonadotropin dose was seen in only three of the five COH protocols when ovarian stimulation was required past cycle day 12. However, the fact that certain protocols were utilized to a lesser degree may limit the ability to meaningfully assess the differential relationship between cumulative gonadotropin dose and aneuploidy according to COH protocol. Future studies with a larger sample size of patients undergoing various protocols may allow for a complete comparison of the effect of using GnRH agonists or antagonists, in conjunction with exogenous gonadotropins, on embryonic ploidy and clinical outcome.

Since the advent of assisted reproduction, the goal of COH has been to optimally stimulate follicular maturation and oocyte yield to overcome the inefficiency of human reproduction, while circumventing patient-specific causes of infertility. Given the lack of association between cumulative gonadotropin dose and embryonic aneuploidy in normal responders, the rationale behind minimalist stimulation techniques that minimize oocyte yield should be questioned. Using a recent, clinically validated PGS platform, this analysis provides reassurance to both clinicians and patients that the cumulative dose of exogenous gonadotropins used for COH does not increase the odds of aneuploidy in patients who exhibit a normal response to COH. Patients that require COH for an extended duration may represent a poor prognosis cohort with an inherent predisposition towards oocyte and embryonic aneuploidy.

Compliance with ethical standards

Research approval was obtained from Western Institutional Review Board, and all subjects provided informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

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